CHARACTERIZATION OF SUBSTRATE UPTAKE BY AVIAN SKELETAL MUSCLE

by

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Dedication

This dissertation is dedicated to my parents, Jean and Robert Sweazea, who have provided support, literally and figuratively, throughout my educational career. I could not have made it this far without their love and faith in me.

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ABSTRACT

The goal of this work was to characterize avian skeletal muscle (SKM) glucose and fatty acid uptake. English sparrows (*Passer domesticus*) were used for the following studies: 1. Characterization of glucose uptake, 2. Identification and localization of glucose transporters, 3. Characterization of free fatty acid uptake, and 4. Reciprocal inhibition of glucose and free fatty acids. The results are summarized as follows. Isolated SKM incubated for 60 minutes with insulin, IGF-1, caffeine or AICAR demonstrated no increase in glucose transport. Interestingly, uptake was decreased in the presence of incremental unlabeled glucose suggesting the presence of glucose transporters (GLUT) and by phloretin, an inhibitor of transport proteins, decreased transport. The SKM glycogen content was low, which is supportive of the observed minimal glucose uptake. These findings suggest that GLUT expression may differ in birds as compared to mammals. GLUT1 and GLUT3 gene expression, but not GLUT4, were found in all tissues examined and share a high degree of homology with published chicken sequences. In addition, GLUT3 and GLUT4 proteins were not detected, whereas GLUT1 protein was abundant in blood-tissue barriers. Sparrows have high plasma ketone body levels suggestive of a high rate of free fatty acid (FFA) oxidation. *In vitro* uptake of radiolabeled oleic acid (OA) was maximal at 60 minutes and competitively inhibited by unlabeled OA suggesting a facilitative process. Radiolabeled OA uptake was not increased by IGF-1, caffeine and AICAR, whereas insulin increased uptake at 60 minutes. Inhibitors of protein-mediated substrate transport increased OA uptake by 60 minutes (DIDS and phloretin) whereas a specific inhibitor of long chain FFA transport,

sulfo-*N*-succinimidyl oleate, decreased its uptake at 2.5 min. In reciprocal inhibition studies, 20mM unlabeled glucose and OA inhibited the uptake of their radiolabeled counterparts. Glucose (20mM) significantly decreased labeled OA uptake 36% and 20mM OA significantly decreased labeled glucose transport by 49%. These data begin to elucidate why avian skeletal muscle may not take up glucose to an appreciable extent and further, why avian skeletal muscle is insulin resistant.

CHAPTER 1

INTRODUCTION

Energy transduction to sustain activity of vertebrates is supported mainly by two classes of metabolic substrates: carbohydrates and lipids, with protein (amino acids) playing a relatively minor role unless the other two substrates have been depleted (Randle, 1998). The focus of the present study is on the uptake of carbohydrates (glucose) and free fatty acids (oleic acid) into avian skeletal muscle cells.

Background

In mammals, insulin is the principle hormone that regulates plasma glucose concentration by stimulating the uptake of glucose into skeletal and cardiac muscle as well as adipose tissue. This is very important following a glucose load that can occur during a regular meal. For humans, insulin keeps the normal range of plasma glucose concentrations between 80 to 100mg/dl. In contrast, the normal levels in birds range from about 300 mg/dl in some domestic animals to 650 mg/dl in small, nectarivorous species (Beuchat and Chong, 1998). When plasma glucose concentrations are plotted against body mass for mammals, an inverse relationship is seen such that small mammals have higher plasma glucose concentrations

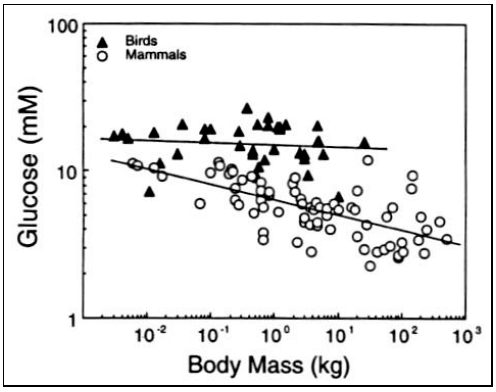


Figure 1.1: Plasma glucose concentration as a function of body mass for birds and mammals. Data from Umminger 1975 and Beuchat and Chong 1998.

than larger mammals (Fig.1.1). When a similar plot is generated for birds, it becomes apparent that plasma glucose concentration is relatively independent of body mass (Fig. 1.1).

Experimental Model

Birds were chosen for the current studies to examine their unique ability to maintain these high plasma glucose concentrations. We were particularly interested in the peripheral utilization of substrates, i.e. glucose and fatty acids, by skeletal muscle. Considering the high plasma glucose levels, avian skeletal muscle may function in a manner different from that of mammals to prevent the lowering of plasma glucose.

Glucose Uptake

Glucose is an essential nutrient for all vertebrates and therefore its concentration in extracellular fluid is closely regulated. This glucose homeostasis depends on a delicate balance between hepatic glucose output and glucose uptake by various tissues (Stumvoll et al., 1997). Mammalian skeletal muscle accounts for about 80% of glucose uptake, with adipose tissue accounting for much less disposal of plasma glucose (Zierath et al, 1996). Thus, skeletal muscle is quantitatively the most important tissue involved in glucose homeostasis. This homeostasis must be maintained to prevent the negative effects of elevated plasma glucose concentrations and to ensure an adequate supply of glucose for the central nervous system. *In vitro* avian skeletal muscle glucose transport has yet to be examined.

Identification/Localization of GLUTs

Glucose is a hydrophilic molecule and, as such, its transport into tissues is facilitated by a family of structurally related proteins encoded by distinct genes (Joost and Thorens, 2001). This is the glucose transporter (GLUT) family of proteins. Thus far 13 of these proteins have been identified (depending on how they are counted or re-named in some cases) (Stuart et al., 2000). Little is known of the identification and localization of the GLUT proteins in avian tissues. Much of the published work in this area has been conducted using chicken embryonic fibroblasts as opposed to whole tissue preparations. The work presented in chapter 3 was conducted to identify the specific GLUT proteins localized to avian skeletal muscle as this tissue is responsible for the majority of postprandial glucose uptake in mammals. GLUT1 and 3 gene expression was examined in a multitude of avian tissues and resultant RT-PCR products were sequenced to identify the specific sparrow GLUT sequences.

Fatty Acid Uptake

Fatty acids provide large amounts of energy for avian muscles, especially during flight. As skeletal muscle cells do not have the capacity to synthesize free fatty acids, these substrates have to be delivered to cells by the circulation. However, it is not clear how these free fatty acids are taken up by avian muscle cells nor how their uptake changes in the face of pharmacological perturbations.

Transport Studies

Little is known of the mechanism of glucose and oleic acid entry into skeletal muscle of birds. English sparrow *extensor digitorum communis* (EDC) skeletal muscles were used for this study to quantify *in vitro* radiolabeled-glucose (Ch. 2) and oleic acid (Ch. 4) uptake using an established protocol adapted from Bonen et al., (1994). In addition, studies on the reciprocal inhibition of glucose and oleic acid are presented in chapter 5.

Hypothesis

The use of metabolic substrates by avian skeletal muscle is qualitatively different from that of mammals such that avian skeletal muscle preferentially utilizes free fatty acids, as opposed to glucose, as a source of energy to sustain muscle contraction.

Specific Aims

1. To examine and characterize glucose uptake by avian skeletal muscle (Ch 2).
2. To characterize English sparrow glucose transporters (Ch 3).
3. To examine and characterize free fatty acid uptake by avian skeletal muscle (Ch 4).
4. To examine the reciprocal inhibition of glucose and fatty acid uptake in avian skeletal muscle (Ch 5).

CHAPTER 2

GLUCOSE TRANSPORT BY AVIAN SKELETAL MUSCLE *IN VITRO*: HAVE WE BEEN CHIRPING UP THE WRONG TREE?

Abstract

Glucose uptake by mammalian skeletal muscle has been extensively covered in the literature whereas the uptake of glucose by avian skeletal muscle has yet to be examined. As skeletal muscle provides the majority of postprandial glucose uptake in mammals, this study was designed to characterize the glucose transport mechanisms and glycogen content of avian skeletal muscle. In addition, plasma glucose levels were measured. English sparrow *extensor digitorum communis* (EDC) skeletal muscles were used for this study to quantify *in vitro* radiolabeled-glucose uptake. Uptake of labeled glucose was shown to decrease in the presence of increasing unlabeled glucose and was maximal by 60 minutes of incubation. Various agents known to increase glucose transport in mammalian tissues, via the insulin and contraction-responsive pathways, were used to manipulate and characterize *in vitro* transport in birds. The typical effectors of the mammalian insulin pathway, insulin (2ng/ml) and insulin-like growth factor-I (48ng/ml), did not increase skeletal muscle glucose transport. Likewise, inducers of the mammalian contraction-responsive pathway had no effect on glucose transport by *in vitro* avian skeletal muscle (5mM caffeine, 2mM AICAR (5'-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside). Interestingly, 200μM phloretin, an agent used to block glucose transport proteins, significantly inhibited its uptake ($P<0.001$). Our results suggest that a glucose transporter is responsible for glucose uptake by avian skeletal muscle, albeit at

unexpectedly low levels considering the high plasma glucose concentrations (265.9 ± 53.5 mg/dl) and low skeletal muscle glycogen content (9.1 ± 4.11 nM glucose/mg) of English sparrows.

Introduction

The majority of what is known of skeletal muscle glucose transport and the action of pharmacological agents to manipulate this process is related to work on mammalian model systems. Much less, in fact very little, is known of the mechanism of glucose entry into skeletal muscle and adipose tissue of birds (Chen et al., 1945; McMurtry et al., 1989; Ashwell and McMurtry, 2001). To our knowledge, the present study is the only examination of *in vitro* glucose transport by avian skeletal muscle.

In humans, skeletal muscle is quantitatively responsible for the majority (80%) of glucose disposal after a meal. This glucose uptake occurs primarily by two mechanisms, namely insulin and contraction induced pathways. The postprandial uptake of plasma glucose into storage tissues occurs primarily by the insulin pathway (Zierath et al., 1996). The contraction-induced pathway is important during exercise bouts to provide energy for working muscles.

In mammals, insulin is released from pancreatic β-cells in response to increased plasma glucose concentrations and is the principle hormone that regulates plasma glucose by stimulating its uptake into skeletal and cardiac muscle as well as adipose tissue. For humans, insulin maintains the normal plasma glucose concentration between 80 to 100mg/dl. If plasma glucose concentrations reach 120 to 150 mg/dl and remain elevated

for four to six weeks, the symptoms of type-2 diabetes will begin to appear (peripheral and retinal vascular damage). Avian plasma glucose levels are much higher than levels observed in mammals of comparable body mass. These normal levels in birds range from about 300 mg/dl in some domestic animals to 650 mg/dl in small, nectarivorous species (Beuchat and Chong, 1995). The rather amazing point is that, despite these high plasma glucose levels, birds do not develop the pathologies of Type-2 diabetes (Klasing, 1998).

Mammalian skeletal muscle expresses receptors for both insulin and insulin-like growth factor (IGF-I). In addition IGF-1 binds to the insulin receptor, or its own receptor, to increase glucose transport (Poggi et al., 1979). Binding of insulin to its receptor increases glucose transport into cells via enhanced translocation of glucose transporters from intracellular storage vesicles to the plasma membrane (Shepherd and Kahn, 1999). The role of insulin in the plasma glucose homeostasis of birds appears less prominent than is the case for mammals, as the hypoglycemic effect of insulin is less severe in birds (Chen et al., 1945; McMurtry et al., 1989; Ashwell and McMurtry, 2001). For domestic fowl, the *in vitro* infusion of glucose leads to a transient spike in plasma insulin concentration such that insulin levels return to baseline in about twenty minutes (Vives et al., 1981; Ruffier et al., 1996, Chida et al., 2000). In addition, a large dose of insulin (22.5U/kg/dy), fatal to mammals of comparable body mass, was required to stimulate plasma glucose disposal in birds.

The insulin-independent pathway for increased glucose transport is activated by muscle contractions and hypoxia. Through a cascade of events, glucose transporters

(GLUTs) are translocated to the plasma membrane by vesicles where they become available for glucose uptake into muscle cells.

There are a multitude of pharmacological agents commonly used to perturb various aspects of the insulin and contraction-induced glucose transport pathways. The purpose of the present study was to utilize these agents to characterize the glucose transport mechanisms in avian skeletal muscle.

Materials and Methods

Experimental Animals and Tissue Preparation

English sparrows (*Passer domesticus*) were captured by mist net on the morning of each experiment at the University of Arizona Dairy Research Center. Birds were anesthetized with 40 µl of sodium pentobarbital (65 mg/ml) and the *extensor digitorum communis* (EDC) muscles extracted. The EDC is a small muscle (~24 mg) between the radius and ulna that has well defined tendons on either end allowing it to be extracted without damage. All animal protocols have been approved by the University of Arizona Institutional Animal Care and Use Committee (IACUC).

Measurement of Glucose in Whole Arterial Blood

Arterial blood samples were collected into heparinized capillary tubes from anesthetized sparrows prior to muscle extraction and placed on ice. Samples of whole blood were analyzed for glucose content using PTS PANELS glucose test strips and read

with a CardioChek P•A meter (Polymer Technology Systems, IN). This instrument determines the quantity of glucose in a sample by reading the change in light reflected off the test strip: the darker the test strip color, the more glucose present in the sample. The meter converts the readings to mg/dl of glucose.

Glycogen Staining of EDC by Periodic Acid Schiff Reaction

For these studies, cryosections (8μm) were made of English sparrow EDC muscles that had been embedded in OCT compound (Tissue-Tek). Sections were collected on glass coverslips which were placed in Coplin jars. The sections were fixed in acetone (5 min), followed by a 5-minute incubation in 0.5% periodic acid to oxidize glucose residues to dialdehydes which were then visualized with Schiff's reagent (15 min). In between incubations, sections were rinsed with DI water. After staining with Schiff's reagent, sections were rinsed in tap water for 10-15 minutes and mounted onto glass slides with Shurmount®. Staining was viewed using a light microscope and images were recorded. Fibers containing glycogen appear dark pink while those that have been depleted of glycogen stain pale pink (Pearse, 1968).

Quantification of Avian EDC Glycogen

Glycogen from EDC muscles (n=12) was precipitated and converted to glucose for analysis according to the methods of Hassid and Abraham (1957). Briefly, EDC muscles were digested in heated 5N KOH. Saturated sodium sulfate and 1.5ml 95% ethanol were then added, the solution was heated to boiling (1-2 min), cooled, and centrifuged at 2000

X g (10 min). The supernatant was discarded and the pellet was dissolved in 1ml heated DI water. To precipitate the glycogen, 1.5ml 95% ethanol was added and the solution boiled, cooled, and centrifuged at 2000 X g for 10 min. After discarding the supernatant, 0.5ml of 2N HCl was added to the pellet which was incubated in boiling water for 2.5-3 hours followed by neutralization to pH 6-8 (using 4N KOH, 0.1M triethanolamine). The volume was adjusted to 2.0ml with DI water and assayed for glucose.

Enzymatic Glucose Assay

Buffer (0.9ml: 50mM triethanolamine, 10mM $MgCl_2$, 5mM EDTA, 0.5mM ATP, 0.48mg/ml NADP, and 5mg/ml G6PDH) and 0.1ml sample were combined and the mixture read spectrophotometrically at 340nm. Hexokinase (10µl) was added to start the enzymatic reaction (20 min). The final readings were made at 340nm and the amount of glucose was determined by the difference between the initial and final readings (Bergmeyer et al., 1981).

Protocol for ALL glucose transport studies.

The uptake of glucose was studied by incubating EDC muscles *in vitro* using a protocol adapted from Bonen et al., (1994). For all transport studies, glucose uptake was quantified using [1,2-^{3}H]-2-deoxy-D-glucose (^{3}H-2-DG) as it is transported into cells, but not metabolized. Excised EDC muscles were incubated in three successive temperature controlled water baths (41˚C, sparrow body temperature) all of which had Krebs Henseleit Buffer (KHB: 0.11M NaCl, 5mM KCl, 2mM NaH_2PO_4, 1mM $MgSO_4$, 25mM

$NaHCO_3$, 1.8mM $CaCl_2$; pH 7.45) as the background solution. The KHB used was the standard buffer solution to which was added several amino acids (5mM L-alanine, 10mM Na-acetate, 1mM Na-citrate, 1.5mM Na-lactate, 1mM Na-malate and 0.9mM glycine). All incubation solutions were continually gassed using 95%O_2:5%CO_2. The total solute concentration for each solution was adjusted to 40mM using mannitol as this concentration maintains an osmolality of 300mOsm/kg-H_2O.

The first incubation solution contained 39 mM mannitol and 1 mM D-glucose and was designed to bring all muscles to the same physiological state. The second solution had 40 mM mannitol and was a rinse to remove any adhering glucose. The final incubation solution (the test solution) contained 300µCi/mM ^{3}H-2-DG and 0.8µCi/mM [U-^{14}C]-mannitol with varying levels of unlabeled substrates which will be described for each experiment. After the final incubation, the individual muscles were rinsed in 2mM phloridzin in KHB, clamp frozen in liquid nitrogen, and weighed (to 0.1 mg). The muscles were then placed in liquid scintillation vials containing 500 µl of 0.5N NaOH and digested in a 60˚C water bath for two hours. To account for background emission from NaOH, a 500µl aliquot of 0.5N NaOH was counted. Scintillation cocktail (EcoLite®) was added to each vial and the samples were dark adapted for three hours prior to counting. Radiolabeled mannitol was used in the final incubation as it is not transported into skeletal muscle and can therefore be used to quantify the extracellular space. The specific activity of an aliquot of labeled glucose was calculated and used to convert the glucose counts to Moles of glucose uptake per mg muscle from which the mannitol counts were subtracted to account for free glucose in the extracellular space.

Time Course of Glucose Uptake

To determine the incubation length at which maximal glucose uptake occurred, a set of glucose uptake experiments at varying final incubation times was carried out. The final incubation solution for the time course studies contained 39 mM mannitol and 1 mM D-glucose and was carried out for 0, 5, 20, 60 or 120 min. For each experiment, two muscles were incubated per time point for a total of n=7.

The Effects of Pharmacological Agents on Glucose Transport

The left EDC muscle from each bird was labeled "control" and was incubated in 1mM D-glucose and 39mM mannitol for the final incubation. The contralateral muscle (experimental) was treated the same as the control up until the final incubation solution at which point the experimental muscles were incubated in the presence of various pharmacological agents (insulin, IGF-I, AICAR (5'-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside), caffeine and phloretin) shown to affect different aspects of the glucose transport mechanism in studies of mammalian skeletal muscle. The concentrations used were chosen as they were comparable to doses used in previous studies on mammalian tissues to elicit a maximal response. All final incubations were carried out for 60 minutes as this time point appeared to be representative of maximal glucose uptake (Fig. 2.2) while minimizing muscle degeneration due to prolonged incubations. Uptake occurring by 120 minutes is likely not physiological and may indicate degeneration of muscle tissue.

Insulin: Chicken insulin (Gift from JP McMurtry) was used to study the responsiveness of sparrow skeletal muscle to this regulatory hormone as insulin from sparrows is not commercially available. Insulin (2ng/ml) was added to the final experimental incubation solution along with 1mM D-glucose and 39mM mannitol. Five birds were used for this experiment providing five control and five experimental muscles.

IGF-1: The uptake of glucose in mammals has been shown to increase in response to IGF-1. This effect may occur via IGF-I binding to the insulin receptor or its own receptor (Poggi et al., 1979; Kern et al., 1989). Human IGF-1 (Sigma) was used in our studies as it displays a high degree of sequence similarity with avian IGF-1 (Ballard et al., 1990). The final incubation solution contained IGF-1 (48ng/ml), 1mM D-glucose and 39mM mannitol. Five paired control and experimental muscles were used for this study.

AICAR: 5'-Aminoimidazole-4-carboxamide-1-b-D-ribofuranoside (AICAR) is an activator of the AMPK pathway and was added to the final *in vitro* EDC muscle incubation preparation. Three paired muscles were used for this study. The final incubation solution contained 2mM AICAR, 1mM D-glucose and 37mM mannitol.

Caffeine: A ryanodine receptor agonist, caffeine, has been shown to increase glucose transport by stimulating the release of calcium from the sarcoplasmic reticulum, thereby mimicking the effects of contraction (Freymond et al., 2002). Muscles extracted from 10 birds were used, giving a total of 10 control and 10 experimental muscles. The final

experimental incubation solution contained 5mM caffeine, 1mM D-glucose and 34mM mannitol.

Phloretin: Phloretin has been shown to inhibit the uptake of glucose in mammalian skeletal muscle preparations (Kasahara and Kasahara, 1997; Teerijoki et al., 2000; Teerijoki et al., 2001) and in myotubes derived from chicken breast muscle satellite cells (Duclos et al., 1993). Phloretin (200 μM) was added to the final preparation which also contained 1mM D-glucose and 39mM mannitol (n=5).

Statistics

The quantity of glucose taken up by the skeletal muscles was expressed as moles glucose uptake per mg muscle (wet weight). The final data for each experiment are shown as means ± SD. Student's T-test was used to determine the level of significance between control and experimental values. $P<0.05$ was the accepted level of significance.

Results

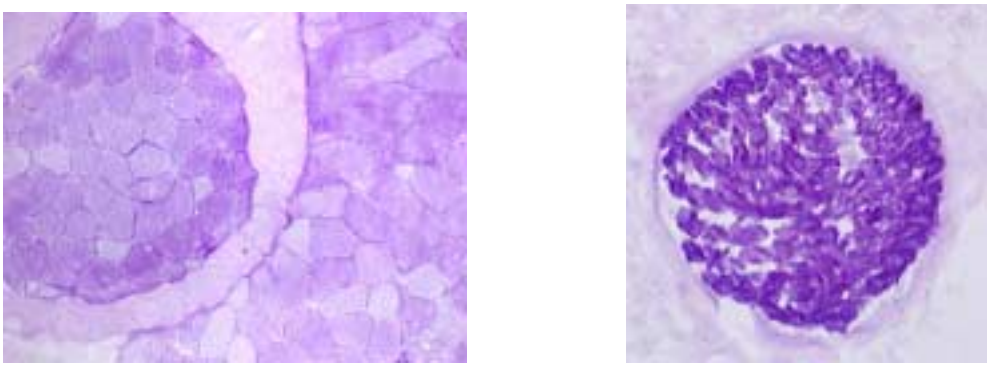
TABLE 2.1. Physiological Data for English Sparrows.

Results are mean values ± SD for all birds. Number of samples (*n*) is indicated in parentheses.

Parameters	**Baseline Data**
Body Mass (g)	24.0 ± 1.53, (91)
EDC Muscle Mass (mg)	23.8 ± 3.65, (182)
Whole Blood Glucose (mg/dl)	265.9 ± 53.5, (18)
EDC Muscle Glycogen (nM/mg wet mass)	9.1 ± 4.11, (12)

Glycogen Staining

Cross-sections (8μm) of EDC muscle were stained for glycogen using the periodic acid schiff reaction. Fibers containing glycogen stained dark pink, while fibers that have been depleted of glycogen stained pale pink. A nerve fiber was observed in the muscle section and, as expected, stained dark pink for the presence of glycogen (Figure 2.1).



A **B**

FIGURE 2.1. Glycogen Staining of EDC Muscle by Periodic Acid Schiff Reaction.

Cryosections (8μm) were made of OCT embedded EDC muscles. Periodic acid oxidizes glucose residues to dialdehydes, which are then visualized with Schiff's reagent. Fibers containing glycogen appear dark pink, while fibers that have been depleted of glycogen stain pale pink. A nerve fiber is shown in panel B and, as expected, stains positively for the presence of glycogen. (A=100X; B=200X)

Time Course of 2DG Uptake

The uptake of ^{3}H-2-DG increased over a 2 hr time course (0, 5, 20, 60, 120 min) in avian EDC muscles (Fig. 2.2). Uptake did not increase significantly between 60 minutes and 120 minutes indicating maximal glucose uptake had been reached (Value at 60 minutes: 9.27e-7 ± 2.18e-7 mol/mg).

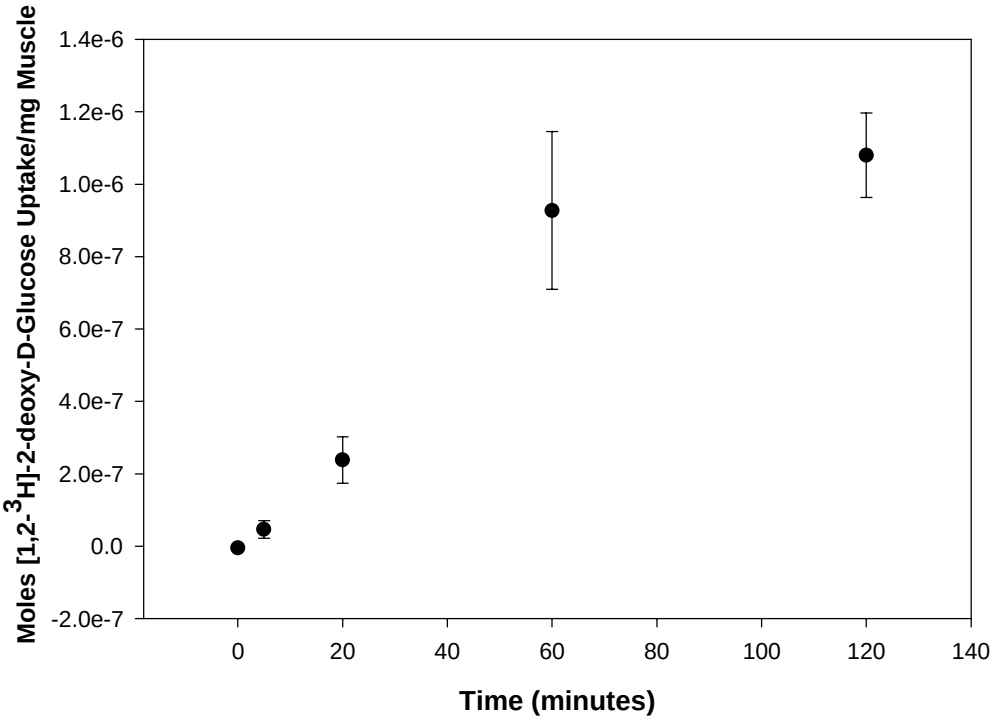


FIGURE 2.2. Concentrative Uptake of Labeled Glucose versus Time in Avian Skeletal Muscle.

Data expressed as Moles [1,2-^{3}H]-2-deoxy-D-glucose uptake per mg muscle (wet weight) ± 1 SD (*n*=7 for each data point). Data show maximal glucose uptake occurred by 60 minutes of incubation.

The Effects of Pharmacological Agents on Glucose Transport

Insulin: Five paired muscles were incubated in either control or experimental conditions. Addition of chicken insulin (2ng/ml) to the final experimental incubation solution had no effect on glucose uptake by the EDC muscle at 60 minutes of incubation in comparison to the controls (Fig. 2.3). Values for the control and experimental muscles were 8.8e-7 ± 1.66e-7 and 8.4e-7 ± 8.8e-8 mol/mg, respectively. These data indicate that sparrow EDC muscle glucose transport are resistant to insulin.

IGF-1: Human IGF-1 binding to the insulin receptor, or its own receptor, results in increased glucose transport in mammals. This agent was added to the final incubation solution (48ng/ml) and the results (Fig. 2.3) show that it had no effect on glucose uptake by avian skeletal muscle (Control: 9.32e-7 ± 2.15e-7; Experimental: 1.02e-6 ± 2.03e-7 mol/mg; *n*=5 paired muscles).

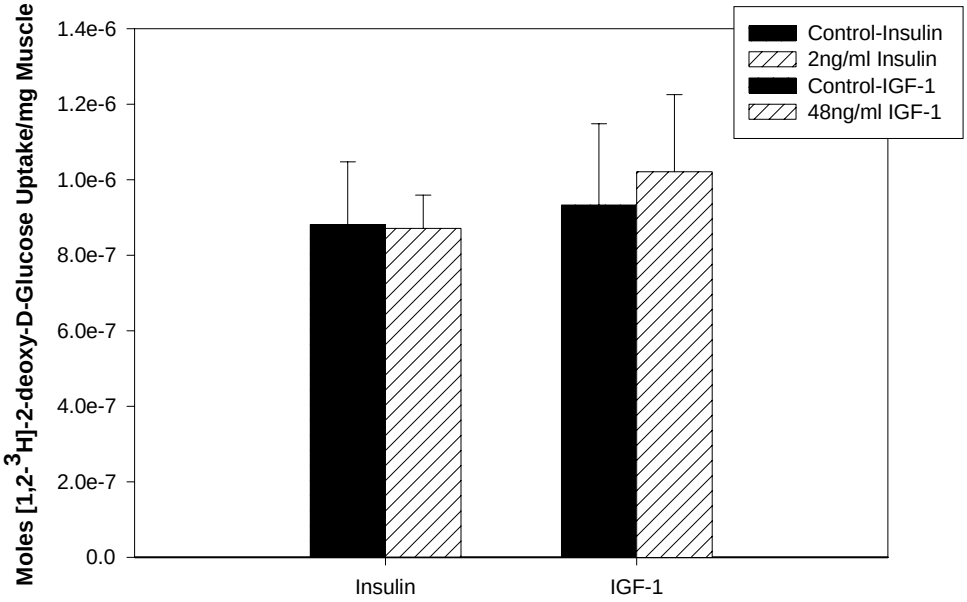


FIGURE 2.3. Effects of Insulin and IGF-I on Glucose Uptake by Sparrow Skeletal Muscle.

Extensor digitorum communis (EDC) muscles were paired and incubated for 60 minutes in the final solution. Control muscles (black bars) displayed similar levels of glucose uptake compared to experimental (striped bars). Chicken insulin (2ng/ml) did not to increase glucose uptake (Control: 8.8e-7 ± 1.66e-7; Experimental: 8.4e-7 ± 8.8e-8; n=5). Likewise, IGF-1 (48ng/ml) which acts through the insulin receptor, and its own receptor, in mammals to increase glucose transport was ineffective at stimulating glucose uptake compared to control muscles (Control: 9.32e-7 ± 2.15e-7; Experimental: 1.02e-6 ± 2.03e-7; *n*=5). Data expressed as Moles [1,2-^{3}H]-2-deoxy-D-glucose uptake per mg muscle (wet weight) ± SD.

AICAR: 5'-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside (AICAR) is known to increase glucose transport in mammalian tissues via increased AMP (as 5'-aminoimidazole-4-carboxamide ribonucleotide (ZMP)) production. Three paired muscles were used for this experiment. Uptake for the control muscles at 60 minutes of incubation (1.07e-6 ± 1.17e-7 mol/mg) did not differ significantly from the experimental incubation containing 2mM AICAR (1.10e-6 ± 1.05e-7 mol/mg). Therefore, AICAR does not appear to significantly increase glucose uptake by avian skeletal muscle (Fig. 2.4).

Caffeine: Stimulation of calcium release in mammals, using caffeine, has been shown to increase glucose transport by mimicking the effects of muscle contraction. This mechanism does not appear to function in birds as labeled glucose uptake was not significantly increased in the presence of 5mM caffeine (Fig. 2.4) (Control: 1.01e-6 ± 1.49e-7; Experimental: 8.94e-7 ± 2.33e-7 mol/mg; *n*=10 paired muscles). In fact, there was a tendency for glucose transport to decrease in the presence of caffeine.

Phloretin: Phloretin is an inhibitor of glucose transport proteins. This substrate was effective at decreasing glucose uptake in avian skeletal muscle at a concentration of 200μM as indicated in Figure 2.4. Uptake of glucose was significantly higher ($P < 0.001$) in the 5 control muscles (1.16e-6 ± 1.13e-7 mol/mg) than for the 5 experimental muscles (5.22e-7 ± 5.76e-8 mol/mg).

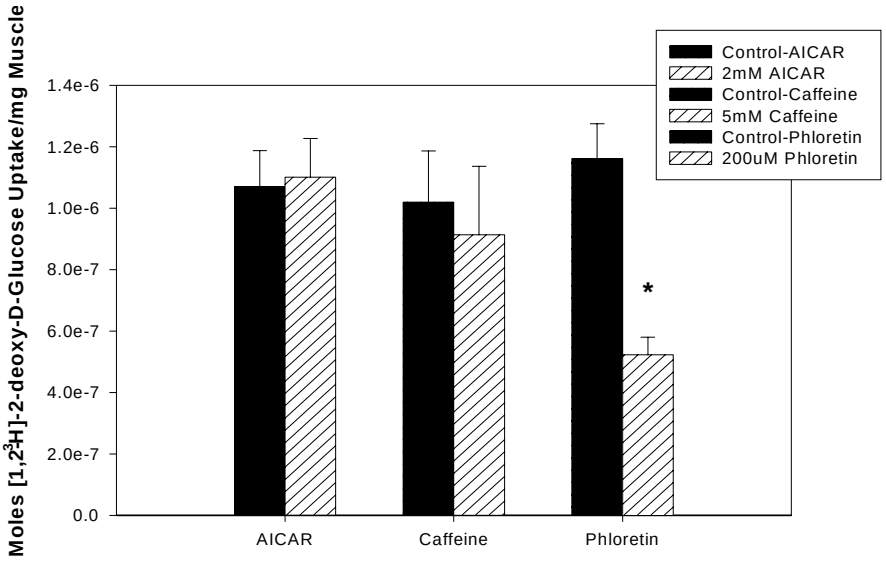


FIGURE 2.4. Effects of Various Pharmacological Agents Known to Stimulate the Contraction Pathway for Glucose Transport.

Paired *extensor digitorum communis* (EDC) were incubated for 60 minutes in the final solution. Data from all control muscles (black bars) show comparable glucose uptake. Experimental muscles are indicated by striped bars. Addition of AICAR (2mM) did not significantly change the quantity of glucose transported (Control: 1.07e-6± 1.17e-7, Experimental: 1.10e-6± 1.05e-7; *n*=3). Likewise, 5mM caffeine had no effect on glucose uptake (Control: 1.01e-6±1.49e-7, Experimental: 8.94e-7± 2.33e-7; *n*=10). Phloretin (200μM), a non-specific inhibitor of glucose transporters, effectively decreased glucose uptake (1.16e-6±1.13e-7, 5.22e-7± 5.76e-8; *n*=5). Values are expressed as mean Moles [1,2-^{3}H]-2-deoxy-D-glucose uptake per mg muscle (wet weight) ± SD (*P<0.001).

Discussion

Birds have very high plasma glucose concentrations (P_{GLU}) in comparison to levels that have been measured for mammals of similar body mass. The P_{GLU} of the birds in the present study averaged 265.9 ± 53.5 mg/dl (14.8 mM/l; Table 2.1). This is very close to the value that can be predicted for a bird with the body mass (24.0 g; Table 2.1) of the English sparrow. Using an allometric equation of plasma glucose and body mass (M) ($P_{GLU} = 15.03M^{-0.014}$; Beuchat and Chong, 1998), a P_{GLU} of 259.2 mg/dl (14.4 mM/l) is predicted for the English sparrow.

Cross-sections of EDC muscle fibers stained positively for the presence of glycogen. The glycogen staining appeared somewhat mottled indicating some fibers had been depleted of glycogen prior to extracting the muscle (Fig. 2.1). A glycogen assay was subsequently performed on EDC muscles to quantify the amount of glycogen present in the whole tissue. Interestingly, the birds in the present study had relatively low levels of intramuscular glycogen (9.1±4.11 nM glucose/mg wet muscle mass; Table 2.1). Data developed for a smaller, summer acclimated bird, the American goldfinch (*Carduelis tristis*) indicated muscle glycogen levels (40.2 nM/mg wet mass) more than four times higher than those of the English sparrow (Marsh, et al., 1990). It is uncertain why the English sparrow levels are so much lower. However, some of the muscle glycogen values measured for winter acclimated goldfinches (10-12 nM) were similar to those of the English sparrow. The birds used in our study were collected in the early summer months. Both birds are resident in their habitats, the goldfinch in the northern temperate

regions and the English sparrows of the present study in the semi-arid southwestern United States.

The transport of glucose into mammalian skeletal muscle cells is controlled predominantly by two mechanisms, namely the insulin and contraction-induced pathways (Fig. 2.5). Very little data exists in the literature describing the intracellular mechanisms involved in avian skeletal muscle glucose transport and, to our knowledge, no data exists examining *in vitro* substrate transport. Therefore, the present study was designed to characterize the putative mechanisms of glucose transport in avian skeletal muscle through the use of pharmacological agents known to affect these pathways in mammalian skeletal muscle. The insulin responsive pathway is stimulated by tyrosine autophosphorylation of the insulin receptor as a result of insulin binding. The ensuing tyrosine kinase activity stimulates insulin receptor substrate -1 (IRS-1), which binds to the SH2 domain of the phosphoinositide-3 kinase (PI3K) p85 subunit. IRS-1 binding to PI3K activates phosphatidylinositol 3,4,5-triphosphate (PIP3), which then activates phosphoinositide dependent protein kinases (PDK), protein kinase B (PKB/Akt) and PKC. These protein kinases, in turn, stimulate the translocation of the insulin-responsive vesicles containing GLUTs to the plasma membrane (Fig. 2.5; Shepherd and Kahn, 1999; Thurmond and Pessin, 2001; Watson and Pessin, 2001; Tomás et al., 2002; Zierath, 2002).

Results from the present study indicate that basal glucose uptake by avian skeletal muscle appears to be minimal, at best (Figs. 2.3, 2.4). In mammals, insulin and IGF-I both bind to the skeletal muscle insulin receptor leading to increased glucose transport. In

addition, IGF-1 acting via its own receptor has been shown to increase glucose transport (Poggi et al., 1979; Dohm et al., 1990). However, mammals resistant to insulin also display skeletal muscle IGF-1 resistance (Pratipanawatr et al., 2002). In the present study, neither of these agents increased glucose transport supporting the findings of others (Dupont et al., 2004) suggesting that avian skeletal muscle is insulin resistant despite expressing IRS-1 and PI3K activity (Fig. 2.3). Chicken muscle satellite cells respond to IGF-1 with increase glucose transport after 4 hour incubation, presumably due to increased protein expression rather than increased transport activity (Duclos et al., 1993).

The insulin-independent pathway of GLUT translocation is triggered by muscle contraction, electrical stimulation and hypoxia (Fig. 2.5). This translocation pathway works through activation of 5'-AMP-kinase (AMPK), which is triggered by increased formation of AMP. AMPK activation increases the quantity of glucose uptake by muscle cells by stimulating the translocation of GLUT-containing vesicles to the plasma membrane in the short term, and in the long term by activating p38 MAPKα and β forming MAPK substrates that activate DNA transcription thereby producing more GLUT proteins (Lemiuex et al., 2003). A pathway distinct from AMPK, also stimulated by muscle contractions, is activated by increases in intracellular calcium released from the sarcoplasmic reticulum (Freymond et al., 2002).

AICAR and caffeine are pharmacological agents widely used to stimulate increased glucose transport in mammals by activating the mechanisms stimulated during muscle contractions. Muscle contractions deplete intracellular ATP levels giving rise to increased AMP which stimulates the phosphorylation of AMPK (Bergeron et al., 1999). AMPK

subsequently stimulates the phosphorylation, and deactivation of acetyl-CoA carboxylase$_{\beta}$ (ACC$_{\beta}$) (Kaushik et al., 2001; Jørgensen et al., 2004) permitting the translocation of GLUT-containing vesicles to the plasma membrane and T-tubules. AICAR, a serine/threonine protein kinase, freely diffuses across the plasma membrane and is phosphorylated by adenosine kinase (Bergeron et al., 1999) to form 5'-aminoimidazole-4-carboxamide ribonucleotide (ZMP), which mimics AMP activation of AMPK (Musi et al., 2001). AICAR has been shown to only induce GLUT translocation to the plasma membrane and NOT to the T-tubules, indicating that it does not mimic all of the pathways by which contraction stimulates the translocation of GLUT-vesicles. Therefore, a pathway distinct from the AMPK pathway must exist for muscle contraction (Lemieux et al., 2003). The results of the present study suggest avian skeletal muscle does not respond to increased AMP levels with enhanced glucose transport (Fig. 2.4).

Inducing calcium release in mammalian skeletal muscle using caffeine, a ryanodine receptor agonist, enhances glucose uptake presumably via stimulation of the second pathway (Freymond et al., 2002). Caffeine, like AICAR, did not increase glucose transport in English sparrow *in vitro* muscle preparation suggesting that avian skeletal muscle does not utilize glucose as an energy source to an appreciable extent for muscle contraction (Fig. 2.4).

Phloretin has been shown to inhibit the uptake of glucose in mammalian skeletal muscle preparations (Kasahara and Kasahara, 1997; Teerijoki et al., 2000; Teerijoki et al., 2001) and in myotubes derived from chicken breast muscle satellite cells (Duclos et al., 1993). In our *in vitro* experimental preparation phloretin (200 μM) was added to the

final incubation solution. The data (Fig. 2.4) show that phloretin is effective at decreasing glucose uptake in whole avian skeletal muscle preparations ($P<0.001$).

Our results indicate that avian EDC muscles transport glucose, albeit minimally, and that this transport is decreased by a glucose transporter antagonist suggesting the presence of a glucose transporter(s). However, agents commonly used to stimulate *in vitro* glucose transport in mammalian skeletal muscle by upregulation of GLUT proteins did not increase transport in avian muscle preparations (Fig. 2.5). The question remains as to which GLUT isoforms are responsible for this facilitative glucose uptake by avian skeletal muscle. Our lab is currently working to identify and localize the glucose transport proteins responsible for the baseline uptake observed in these experiments as well as identifying potential alternative sources of energy.

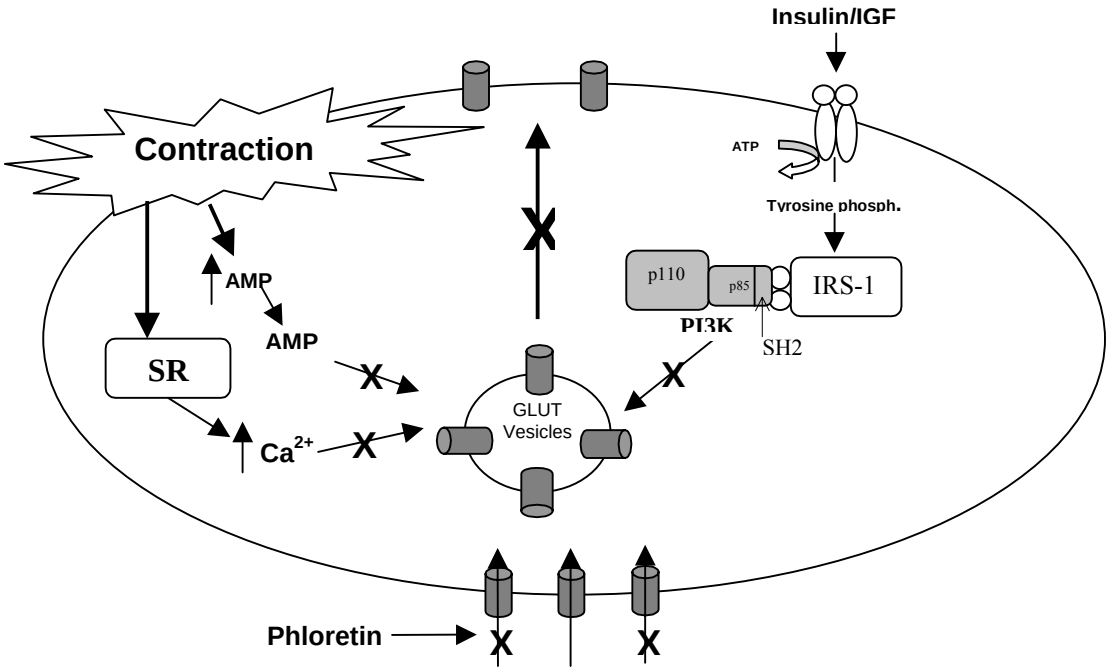


FIGURE 2.5. Schema Adapted from the Literature Depicting Glucose Transport in Mammals with Modifications to Include Data Obtained from Studies using Avian Skeletal Muscle.

Pathways in avian skeletal muscle that may be down-regulated, or nonexistent for glucose uptake, are indicated by "**X**". The mammalian insulin-sensitive pathway is activated by tyrosine autophosphorylation of the insulin receptor as a result of Insulin or IGF-1 binding (Poggi et al., 1979; Dohm et al., 1990). The ensuing tyrosine kinase activity stimulates IRS-1, which binds to the SH2 domain of the phosphoinositide-3 kinase (PI3K) p85 subunit. IRS-1 binding to PI3K ultimately stimulates the translocation and insertion of vesicles containing glucose transporters to the sarcolemma where they become available for glucose transport (Shepherd et al., 1999). The results of our study indicate that this pathway may serve a purpose distinct from glucose uptake in birds. The insulin-independent pathway of GLUT vesicle translocation in mammals is triggered by muscle contraction, electrical stimulation and hypoxia. Muscle contraction degrades ATP, thereby increasing AMP. Increased AMP activates 5'-AMP-kinase (AMPK), which ultimately leads to vesicle translocation by deactivating acetyl-CoA carboxylase (ACC_{β}) (Bergeron et al., 1999; Kaushik et al., 2001; Jørgensen et al., 2004). AICAR stimulates this pathway in mammals (Jørgensen et al., 2004), but was shown to be ineffective at up-regulating glucose transport in birds. Therefore, the AMPK pathway may also serve a purpose distinct from glucose transport in birds. Likewise, calcium (a ryanodine receptor agonist) had no effect on glucose uptake by this muscle. Phloretin significantly decreased glucose uptake when included in the final incubation suggesting that a basal-type glucose transporter exists in avian skeletal muscle which is not inducible by the pharmacological agents used in this study.

Concluding Remarks

Avian skeletal muscle glucose transport appears to be resistant to agents (insulin, IGF-1, AICAR, caffeine) that have been used to enhance transport in mammalian tissues. However, phloretin decreased uptake in the EDC muscle. These results suggest that a glucose transporter is responsible for glucose uptake by avian skeletal muscle, albeit at unexpectedly low levels considering the high plasma glucose concentrations of English sparrows. Furthermore, preliminary studies suggest that glucose transport proteins are expressed in avian skeletal muscle and may be responsible for what little glucose transport is observed.

CHAPTER 3

GLUCOSE TRANSPORTER (GLUT) EXPRESSION IN ENGLISH SPARROWS (*PASSER DOMESTICUS*)

Abstract

Birds are considered hyperglycemic as they display plasma glucose concentrations 3-7 times those of mammals of comparable body mass. The current study was designed to examine some of the possible underlying causes of this difference. To this end, we examined protein and gene expression of various glucose transporters (GLUTs) in select tissues from English sparrows using western blots, immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR). The hypothesis is that the expression pattern of avian GLUTs differs significantly from mammals to maintain the observed high plasma glucose levels. Our results, like those of others, failed to identify an avian GLUT4-like protein or gene. GLUT1 gene expression was identified in all avian tissues examined and shares 88% homology with chicken and 84% homology with human GLUT1. Compared to the rat control, sparrow extensor digitorum communis muscle GLUT1 immunostaining was weak and localized only to highly vascularized areas whereas immunostaining of sparrow gastrocnemius muscles were comparable to rat controls. Gene expression of GLUT3 was identified in all tissues examined and shares 90% gene sequence homology with chicken embryonic fibroblast and 75% homology with human GLUT3. Protein expression of GLUT3 could not be determined as an avian

antibody does not exist and the antibodies used were not specific to the sparrow protein. The predominant difference of avian GLUT expression patterns from that of mammals is the lack of an avian GLUT4. The absence of this insulin responsive GLUT may be a cause of the observed high blood glucose levels as GLUT1 and 3 expression appear to be comparable to that of mammals.

Introduction

Glucose is a hydrophilic molecule that is transported into cells along its electrochemical gradient by a family of structurally related facilitative transporters encoded by distinct genes (Joost and Thorens, 2001). This is the GLUT family of proteins. Thus far 13 of these proteins have been identified in mammalian tissues (depending on how they are counted or re-named in some cases). These transport proteins are composed of 12 transmembrane spanning domains with cytosolic amino and carboxy-termini.

GLUT1, a constitutive plasma membrane transporter present in a wide variety of tissues, is responsible for a basal level of glucose transport. It is highly expressed in brain, erythrocytes, and endothelial cells suggesting its importance in the transport of glucose across blood-tissue barriers, especially the blood-brain barrier (Uldry and Thorens, 2004; Shepherd and Kahn, 1999). Recent data show GLUT1 mRNA expression in heart, gastrocnemius and pectoralis tissues of posthatch chicks; although, studies were not carried out using adult birds (Humphrey et al., 2004). With respect to cDNA, chicken GLUT1 shares 79% identity with mammalian GLUT1 and 95% protein sequence

similarity (Wagstaff et al., 1995). In contrast, mammalian GLUT1s (bovine, human, mouse, rabbit, rat, sheep, and swine) share 97% amino acid sequence identity (Teerijoki et al., 2000).

GLUT3 is a high affinity transporter and, in humans, its mRNA is expressed ubiquitously, although protein expression is found only in brain, testes and skeletal muscle (Haber et al., 1993; Stuart et al., 1999). Protein expression of GLUT3 has been identified in chicken embryo fibroblasts (CEF). Expression of the CEF GLUT3 (CEF-GT3) cDNA isoform in rat fibroblasts resulted in enhanced glucose uptake demonstrating that the CEF-GT3 gene encodes a functional glucose transporter (White et al., 1991).

Phylogenetic analyses show the GLUT4 transport protein to display a great deal of evolutionary conservation. For example, the GLUT4 transporter of brown trout shares greater than 85% amino acid sequence homology with human GLUT4 (Planas, 2000).

In mammals, GLUT4 is the insulin-responsive transporter. Insulin binding to its receptor on target tissues results in the insertion of the GLUT4 transport protein into the cell membrane, from sub-membranous storage vesicles, where it is available to facilitate glucose movement into cells (Shepherd and Kahn, 1999). Other evidence indicates GLUT4 transporters can be inserted into the cell membrane in response to contractile activity. This pathway is independent of the insulin pathway and these GLUT4 proteins originate from an intracellular vesicular pool distinct from the insulin sensitive pool (Douen et al., 1990; Cartee et al., 1991; Coderre et al., 1995).

GLUT8, an insulin-responsive transporter, has been detected in mammalian skeletal muscle, but RT-PCR analyses of avian skeletal muscle failed to identify the presence of

this transporter (Seki, 2003). Therefore, GLUT8 will not be discussed further with respect to avian skeletal muscle in the present study.

Much of what is known of the GLUT transport proteins is related to work on mammalian model systems. Much less, in fact very little, is known of the mechanism of glucose entry into skeletal muscle and adipose tissue of birds. Moreover, from what can be gleaned in the published literature, there is little consensus as to the action of insulin in birds as well as the presence of a GLUT4-like protein (Chen, 1945; McMurtry et al., 1989; Ashwell and McMurtry, 2002).

As much of the work studying GLUT proteins in birds has been done using CEFs, the purpose of this study was to identify and localize the GLUTs in adult English sparrow tissues using western blots, immunohistochemistry and RT-PCR. Select tissues; skeletal muscle (extensor digitorum communis (EDC), pectoralis, gastrocnemius), kidney, heart, and brain were used for this study. The EDC, a forewing muscle, is used to make fine adjustments during flight. This muscle was chosen as previous work in our laboratory focused on the characterization of glucose transport by this tissue (Sweazea and Braun, 2005). Sparrow pectoralis and gastrocnemius were selected to contrast the EDC as they are functionally different. Pectoralis is a major flight muscle, whereas the gastrocnemius functions as a lower leg extensor and digit flexor. Heart and brain tissues were used as controls for the presence of GLUT1 as they contain an abundance of this protein in mammals. Kidney was chosen as a negative control for the presence of GLUT4, but as a positive control for GLUTs 1 and 3.

Materials and Methods

Animals:

English sparrows (*Passer domesticus*) were captured at The University of Arizona Dairy Research Center (Tucson, AZ) by mist net. After anesthetization with 40 µl sodium pentobarbital (65mg/ml), tissues were extracted as indicated. All animal protocols were approved by the University Institutional Animal Care and Use Committee.

Western Blots:

To examine GLUT4 protein expression, *extensor digitorum communis* (EDC) muscles were homogenized in 0.25mL Krebs-Henseleit buffer using a tissue tearer (Model 398, Biospec Products Inc., Bartlesville, OK). Acrylamide gels (10%) were loaded with 25µg protein/lane and run at 200V for 38mins. Rainbow^{TM} molecular weight marker (15 µL; RPN756, Amersham Biosciences, Piscataway, NJ) was used as it provides a visible band at ~43kDa, the approximate molecular weight of the GLUT4 protein.

The protein was then transferred to nitrocellulose membranes by electrophoresis (100V for 90mins). The membranes were blocked with 5% nonfat milk and incubated in GLUT4 antisera (1:600, Santa Cruz Biotechnologies, Santa Cruz, CA) followed by incubation with biotinylated anti-rabbit IgG (1:5000, Santa Cruz Biotechnologies, Santa Cruz, CA). ABComplex/HRP (K0355, DAKO, Carpinteria, CA) was then added (1:50) and the membranes were incubated in enhanced chemiluminescence (ECL) Western Blotting Analysis System (RPN2109, Amersham, Piscataway, NJ) according to the manufacturer's directions. The nitrocellulose membranes were then exposed to X-ray

film. Rat heart tissue homogenate served as a positive control as mammalian cardiac tissue contains an abundance of GLUT4 protein. As expression of GLUTs1 and 3 have previously been identified in avian tissues, western blots were not run for these proteins.

Immunohistochemistry:

Cryosections were made of English sparrow EDC, gastrocnemius and rat soleus muscles that had been embedded in Tissue-Tek® O.C.T. Compound (Miles Inc., Elkhart, IN). Frozen sections (6-10μM) were collected on VWR Superfrost Plus Precleaned slides. A set of control morphology sections were prepared, the first, of which, was stained with hematoxylin to localize nuclear material. For the second control, India ink was injected into the brachial artery of the wing prior to muscle extraction to localize intramuscular capillaries. The final morphology control was a combination of India ink injection and hematoxylin staining.

Following acetone fixation for 5 mins at 4˚C, the tissue sections were permeabilized using Triton X-100 (0.2% in TBS) and endogenous peroxidase was blocked using 1.6% hydrogen peroxide. The sections were then incubated with polyclonal antibodies against the 20 amino acid carboxy-termini of mammalian GLUT1 (AbCam, Cambridgeshire, UK) and GLUT3 (Santa Cruz Biotechnologies, Santa Cruz, CA) as avian antibodies were not commercially available. GLUT4 localization was not examined as western blots were unsuccessful with the available antibodies. Primary antibodies were detected using a horseradish peroxidase labeled secondary antibody and DAB chromogen system detection kit (ABC multi-species complete detection kit (HRP/DAB), AbCam,

Cambridgeshire, UK). This kit also included aqueous hematoxylin for counterstaining. Controls for these studies were preparations with no primary, and no secondary, antibody to bring out nonspecific antibody binding. Sections were visualized using an automated light microscope.

Studies using FITC-conjugated antibodies (immunofluorescence) were abandoned as the EDC muscle demonstrated significant autofluorescence at all wavelengths examined (DAPI (475nm), FITC (528nm), Texas Red (617nm) and CY5 (685nm) (data not shown).

RT-PCR:

Primers

All oligonucleotide primers were synthesized by IDT (Integrated DNA Technologies, Inc., Coralville, IA). As a control, primers for chicken D-glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a housekeeping gene, were designed from the published sequences of chicken GAPDH (sequence not available for English sparrows). The forward primer sequence for GLUT4a is misprinted in Tsirka et al., (2001). Therefore, this primer sequence was redesigned from the sequence available in GenBank (Accession# x14771). All primers sequences are listed in Table 3.1.

TABLE 3.1. Sequences of Oligonucleotides.

Primer Pair	Forward Sequence	Reverse Sequence
ChkGAPDH[a]	5'-ACGCCATCACTATCTTCCAG-3'	5'-CAGCCTTCACTACCCTCTTG-3'
ChkGLUT1[b]	5'-CCACGAACCCGAAGATGGCAACGATG-3'	5'-CCGCTTCCTGATCAACCGCAA-3'
ChkGLUT3[c]	5'-CTTTGTGGCCCTTTTTGAGA-3'	5'-ATCTCCACCATGGGGTTCTT-3'
GLUT4a[d]	5'-GGCCATCTTCTCTGTGGGTG-3'	5'-ACGATGGCCAATTGGTTGAG-3'
GLUT4b[e]	5'-CTGGGTAGGCAGGGTCCTGGGG-3'	5'-GCAAGGACAGTGGACGCTCTCTTTC-3'

[a]Croissant et al., 2000
[b] Möller and Kummer, 2003
[c]University of Delaware
[d]Tsirka et al., 2001
[e]Alquier et al., 2001

GLUTs:

RNA was isolated from EDC, pectoralis, and gastrocnemius muscles as well as heart, kidney, and brain tissues using TRIZOL reagent following the manufacturer's protocol (Invitrogen, Carlsbad, CA).

Unless otherwise noted, chemicals used to transcribe cDNA were purchased from Invitrogen (Carlsbad, CA). Total RNA (4 ug) was reverse transcribed using Superscript III RT, Oligo dT, RNase OUT, and 10mM dnTP mix, which was custom-made according to manufacturer's protocol.

The PCR amplification reaction (25µL) was performed by mixing 20pM forward primer, 20pM reverse primer, 2µl cDNA and 12.5 µl 2X PCR master mix buffer (Promega, Madison, WI). PCR reactions were carried out in a PTC-200 Peltier Thermal Cycler (MJ Research, Waltam, MA). The thermal cycle profile used was 40 cycles of denaturation at 95˚C for 5 min, annealing for 1min and extension at 72˚C for 45 sec. The annealing temperatures used were as follows: GLUT1 58°C, GLUT3 53°C, GLUT4a 57°C, and GLUT4b 63°C. PCR fragments were analyzed by electrophoresis on 1.5% agarose gels and visualized with ethidium bromide and UV light. The predicted product sizes were: ChkGLUT1 500bp, ChkGLUT3 345bp, GLUT4a 288bp, and GLUT4b 645bp.

Gel Extraction of PCR Products:

GLUT3 PCR fragments were extracted from the agarose gels and purified using the Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA) according to manufacturer's

protocol. The amount of cDNA in extracted bands was determined using a NanoDrop® ND-1000 UV-Vis Spectrophotometer and GLUT3 samples were submitted to the University of Arizona Genomic Analysis and Technology Core to be sequenced. The GLUT1 and 4 PCR fragments were submitted to the Arizona State University School of Life Sciences DNA Laboratory to be purified and sequenced.

Results

Western Blots:

Using western blot analyses, a GLUT-4 like protein could not be identified in avian tissue. As expected, a 43 kDa protein was observed in rat heart tissue using GLUT4 antibody (Fig. 3.1).

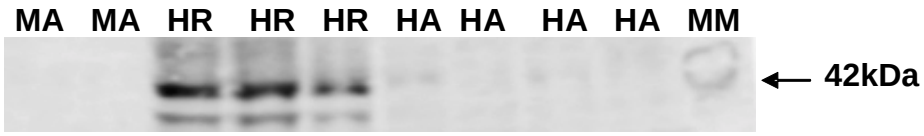


FIGURE 3.1. Western Blot of GLUT4 Protein Expression.

Using an antibody designed against the mammalian GLUT4 sequence, no bands are seen for avian muscle (MA) or heart tissues (HA). Rat heart homogenates (HR) stained positively for the presence of a 42kDa protein. Amersham Rainbow^{TM} molecular weight marker (MM) is shown in the far right lane.

Immunohistochemistry:

The immunostaining patterns for GLUT1 were strikingly different between avian EDC and gastrocnemius muscles. The EDC muscles, shown in Fig. 3.2b, were immunostained in areas associated with capillaries and within nerves (data not shown) whereas immunostaining of the gastrocnemius revealed a more heterogeneous pattern (Fig. 3.2c). The immunostaining pattern observed in the gastrocnemius was comparable to that observed in rat soleus muscle (Fig. 3.2d). GLUT3 immunostaining was not distinguishable by this method as no apparent difference existed between the control and experimental sections (data not shown). These results suggest that either: the GLUT3 protein is expressed at a level too low to be detected by this method, or the avian GLUT3 transporter differs significantly from the mammalian GLUT3 such that the antibody does not recognize the avian isoform.

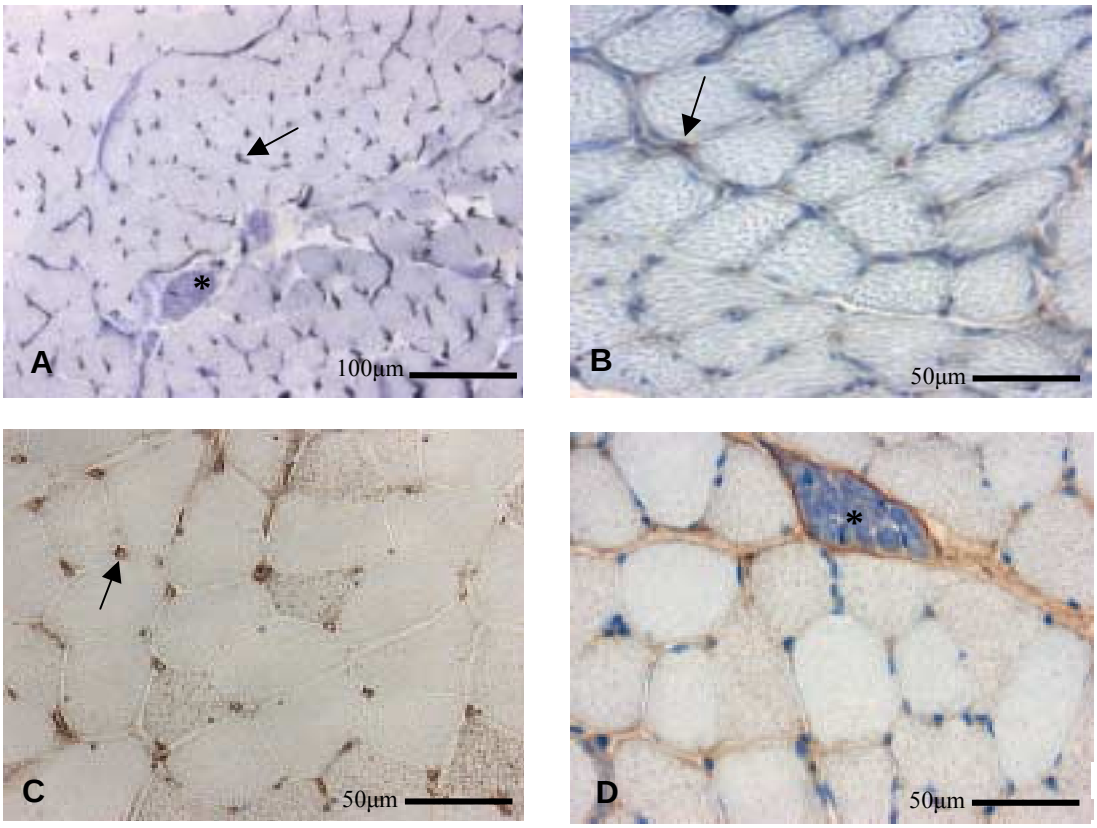


FIGURE 3.2. Histochemical Cross Sections of English Sparrow and Rat Skeletal Muscles.

Panel A) India ink was injected into the brachial artery to localize intramuscular capillaries. Hematoxylin was used to co-stain nuclear material within the muscle fibers (200X). Panel B) GLUT1 immunostaining shows brown punctate staining in highly vascularized areas (400X). Panel C) Cross section of English sparrow gastrocnemius viewed at 400x showing GLUT1 immunostaining of fibers. It is evident that the heterogenous fiber types of this muscle contain varying amounts of GLUT1 protein. GLUT1 immunostaining is evident in areas with abundant vascularization and immunostaining can be seen in circular patterns, indicating capillary presence, between muscle fibers. It is evident from the pattern that this muscle is made up of heterogeneous fiber types as some fibers are surrounded by more capillaries than others. Panel D) Cross section of Sprague-Dawley rat soleus muscle viewed at 400x. Sections were immunostained using the GLUT1-HRP method (refer to text for detailed methods). Various fiber types within the rat muscle display different levels of staining depending on the amount GLUT1 protein. Dark staining is evident in the perineurial sheath, an area known to stain intensely for GLUT1 in rat skeletal muscle (Handberg et al., 1992) (Asterisks: Nerves; Arrows: blood vessels).

RT-PCR:

GLUT1 gene expression was found in the EDC, gastrocnemius, pectoralis, heart, kidney, and brain tissues of the English sparrow (Fig. 3.3a). A partial sequence of sparrow GLUT1 was determined by isolation and sequencing of the bands. The English sparrow GLUT1 sequence shares 88% homology with chicken and 84% homology with human GLUT1 genes (Fig. 5).

Using the primers designed from the chicken CEF-GT3 sequence, PCR products were observed in all tissues examined (Fig. 3.3b). A partial sequence of sparrow GLUT3 was elucidated by isolation and sequencing of the bands. The sparrow GLUT3 cDNA shares 90% identity with chicken embryonic fibroblast and 75% with human GLUT3 (Fig. 6).

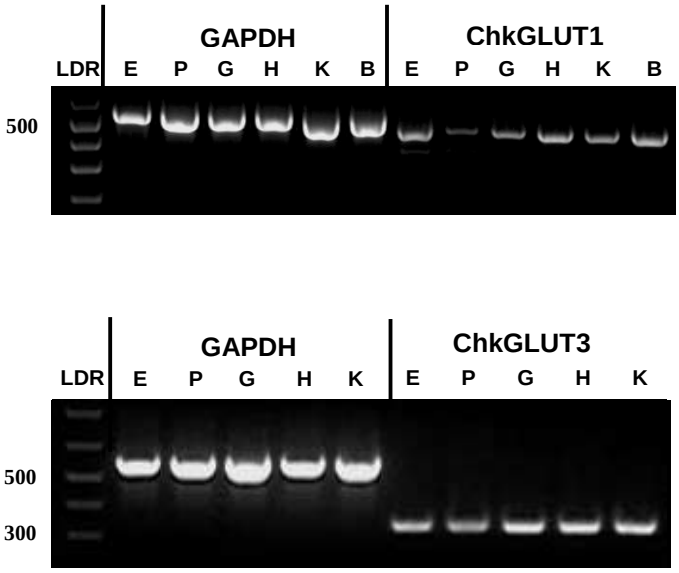


FIGURE 3.3. Agarose Gels (2%) of GLUT1 and 3 PCR Products.

Experimental cDNA was obtained from EDC (E), pectoralis (P), gastrocnemius (G), heart (H), kidney (K), and brain (B) tissues. Panel A. Chicken GAPDH was used as a positive control (585 bp). GLUT1 primers designed from the published chicken sequence (Möller and Kummer, 2003) were used for each of these tissues. Using the chkGLUT1 primers, a 450 bp product was visible in all tissues. Visible bands were extracted, purified, and sequenced. Panel B. Chicken GAPDH control primers were used as a positive control (585 bp). ChkGLUT3 primers designed from the published chicken genome were used. PCR products (345 bp) were shown for all tissues examined. Visible cDNA bands were extracted, purified and sequenced.

GLUT4 mRNA content of sparrow tissues was examined as results from Western blots indicated that GLUT4 protein is not expressed in avian tissues (Fig. 3.1). Distinct bands (585 bp) were seen for the positive controls (GAPDH) of all tissues examined (Fig. 3.4). GLUT4a and GLUT4b primers were used for EDC, pectoralis, gastrocnemius, heart, and kidney cDNA. PCR products (~500bp) formed using GLUT4a primers but were well above the 288bp expected product size. No PCR products were formed using GLUT4b primers. Based on the results of these studies and those of others, it is likely that GLUT4-like mRNA is not expressed in avian skeletal muscle.

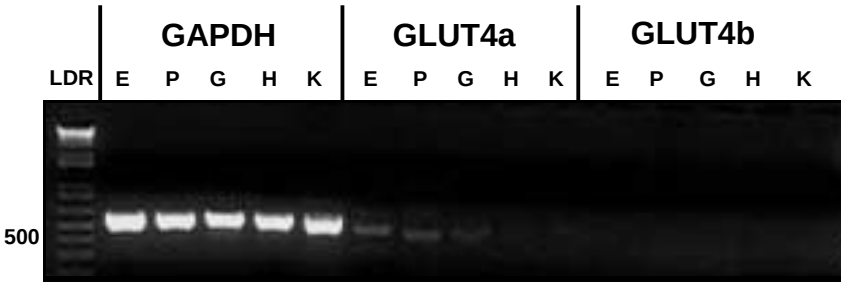


FIGURE 3.4. Agarose Gel (2%) of GLUT4 PCR Products.

Lane 1 is the 1kb DNA Ladder. GAPDH was used as a positive control for EDC (E), pectoralis (P), gastrocnemius (G), heart (H), and kidney (K) cDNA. GLUT4a and GLUT4b primers were used for these tissues. The predicted product size for GLUT4a was 288bp and GLUT4b was 645bp. PCR products were seen at approximately 500bp using GLUT4a primers, which is well above the predicted 288bp product size. Sequencing analysis proved unsuccessful at determining the identity of these bands. No PCR products were seen using GLUT4b primers.

```
Sparrow GLUT1 22   tttggtcnngcagcgtgagggcgatggtcataagaatggcacacccagccatgcccgcca 81
                   |||||||  ||||||| || || |||||||| || || |||||||| ||||| || ||||
ChkGLUT1      1130 tttggtccagcagcgtcagagcaatggtcatgagtattgcacaccctgccatccctgcca 1071


Sparrow GLUT1 82   gcccaatgaggtgcagggtcctgcgtccggctctctccaccacgaagagtgagaccactg 141
                   | |||||||||||| |||||||||||||| |||| |||||||||||||| || || || |
ChkGLUT1      1070 ggccaatgaggtgaagggtcctgcgtccagctcgctccaccacgaagagcgaaacaacag 1011


Sparrow GLUT1 142  tgaaggctgtgttcaccacgccagagccaatggtggcatagacaggctgctccactcccg 201
                   |||| ||||| |||||||| |||||||||||||||| ||||| |||||||||||| || |
ChkGLUT1      1010 tgaaagctgtattcaccaccccagagccaatggtggcgtagacgggctgctccacacctg 951


Sparrow GLUT1 202  acttctcnaagatgctggtggagtagtagaagacagcattgatccctgagagctgctggg 261
                   ||||||| |||||||||||||||||||||| || ||||||||||||||||||||||||||
ChkGLUT1      950  acttctcaaagatgctggtggagtagtagaaaaccgcattgatccctgagagctgctggg 891


Sparrow GLUT1 262  agagctgcaggacaatggcgatgaggatgggctggcggtacatgggcgagcggaacagct 321
                   | ||||||| ||| || |||||||||||||||||||||||||||||| |||||||| ||||
ChkGLUT1      890  acagctgcaagacgattgcgatgaggatgggctggcggtacatgggagagcggaagagct 831


Sparrow GLUT1 322  ccatgatggtgaccttcttctccctcatcatctgccggctctcctccttcatctcctgca 381
                   |||| || |||||||||||||| |||||||| ||||||||||||||||||||||||||||
ChkGLUT1      830  ccattatagtgaccttcttctctctcatcatttgccggctctcctccttcatctcctgca 771


Sparrow GLUT1 382  ggtcgctgctcacgtccgtcgtgcctcgcagcttcttgaggacactcttggctttgttct 441
                   | |||||||| || || || ||||| || |||||||||||||| ||||||||||||||||
ChkGLUT1      770  gatcgctgctgacatctgttgtgccccggagcttcttgaggacgctcttggctttgttct 711


Sparrow GLUT1 442  cctcgttgcggttnatc 458
                   |||| |||||||| |||
ChkGLUT1      710  cctcattgcggttgatc 694
```

FIGURE 3.5. Alignment of Partial English Sparrow GLUT1 with the Published Chicken Sequence.

The deduced amino acid sequence is listed below the cDNA sequences. BLAST sequence homology shows 88% identity with chicken GLUT1 (ChkGLUT1; GenBank Acc# NM_205209).

```
Sparrow GLUT3  14   attgtggcagaactcttcagccagggcccacgacctgcagccatggccgtggctggttgt 73
                    ||||||||||||||| ||||| || |||||||| ||||||||||||||| ||||||||||||
CEF-GT3        1242 attgtggcagaacttttcagtcaaggcccacggcctgcagccatggcggtggctggttgt 1301

Sparrow GLUT3  74   tccaactggacctccaatttcttggtgggaatgctcttcccctatgcagagaaactgtgt 133
                    || |||||||||||| |||||||| |||||||||||||||||||||||| |||||| | |||
CEF-GT3        1302 tctaactggacctctaatttcttagtgggaatgctcttcccctatgctgagaaattatgt 1361

Sparrow GLUT3  134  ggctcctatgtcttcctcatcttccttgttttcctggtcatcttctttgtcttcaccttc 193
                    ||| |||||||||||||| |||||||||||||||||| ||||||||||  | ||||| | |
CEF-GT3        1362 ggcccctatgtcttccttatcttccttgttttcctgctcatcttcttcatattcacatac 1421

Sparrow GLUT3  194  ttcaaagtgccagagaccaggggcaggacttttgaagacatctccagaggctttgaagga 253
                    |||||||||||||||||| |||||||||||||||| |||||||| |||||||||| |
CEF-GT3        1422 ttcaaagtgccagagaccaagggcaggacttttgaagatatctccaggggctttgaagaa 1481

Sparrow GLUT3  254  caag 257
                    ||||
CEF-GT3        1482 caag 1485
```

FIGURE 3.6. Alignment of Partial English Sparrow GLUT3 cDNA Sequence with Chicken Embryonic Fibroblast GLUT3 (CEF-GT3).

The deduced amino acid sequence is listed below the cDNA sequences. BLAST sequence homology shows 90% identity with CEF-GT3 (Univ. Delaware Database).

Discussion

This study was designed to characterize GLUT protein and gene expression in adult birds of flight, English sparrows, as the majority of work done in this area has been conducted using chicken embryonic fibroblasts (Duclos et al., 1993; Carver et al., 2001). Data in the literature question whether GLUT4, the mammalian insulin responsive transporter, is present in avian skeletal muscle. To date, there is one published report of a GLUT4-like protein being present in avian muscle (Thomas-Delloye et al., 1999) and several reports contrary to this finding (Duclos et al., 1993; Carver et al., 2001; Seki et al., 2003). Using an antibody against rat GLUT4 and membrane vesicles prepared from duckling gastrocnemius muscle, a Western blot illustration showed a band at about 47 kDa, the expected mass of a GLUT4 protein (Thomas-Delloye, et al., 1999). However, twice the amount of avian total protein as compared to the rat control had to be loaded on the gel for a band to be detected by the antibody to the rat protein. Moreover, no attempt was made to sequence the protein that migrated to 47kDa position on the gel. Without sequence information one cannot be certain of the identity of the protein as the various GLUT protein isoforms share a degree of sequence similarity. In addition, all GLUT proteins have a molecular mass of about 45-55 kDa (depending on the degree of glycosylation) and there is a degree of cross reactivity among the antibodies to the various GLUT proteins.

Three other published studies using immunological techniques have failed to detect the presence of a GLUT4-like protein in avian muscle. Duclos, et al. (1993) failed to identify a GLUT4-like protein in selected chicken tissues, but they were able to detect the

presence of GLUTs-1 and 3. Using antibodies against two distinctly different epitopes of the rat GLUT4 protein Duclos states, "no immunostaining was observed in any chicken tissue tested including muscle and adipose tissue" (Duclos et al., 1993). In another aspect of these studies, the uptake of ^{3}H-deoxyglucose was found to be linear with time and was completely blocked by cytochalasin B and phloretin. These results indicate that facilitated transport was occurring and that it was inhibitable by compounds that block glucose transport in mammalian skeletal muscle cells. The suggestion is that GLUTs-1 and 3 were responsible for the glucose uptake.

A second published study failed to detect the presence of GLUT4 mRNA or protein in avian skeletal and heart muscle despite using four different polyclonal GLUT4 antibodies (anti-rat and anti-human; Carver et al., 2001). As a control to the developing chick, adult chicken tissues (leg and breast skeletal muscle, brain, liver, heart and adipose) were examined and, again, no GLUT4 expression was detected. This same study demonstrated the presence of GLUTs-1 and 3 in the tissues of the developing chick. However, tissue from adult birds was not examined for the presence of GLUTs-1 and 3.

A third study performed by Seki et al., (2003), showed the lack of GLUT4 expression as demonstrated by northern blot analyses of various chicken tissues. Moreover, our data suggest that mRNA and protein encoding an avian GLUT4 are not present in skeletal or cardiac muscle extracted from English sparrows (Figs 3.1, 3.4). Taken together, it can be concluded that avian muscle does not express GLUT4-like protein or mRNA. These

findings are further supported by the lack of an identified GLUT4 sequence in the published chicken genome database (Univ. Delaware Database).

Western blots were not run for GLUTs1 and 3 as these proteins have been identified in avian tissues (Duclos et al., 1993; Carver et al., 2001). However, these studies did not attempt localization of these proteins in adult avian skeletal muscle. In the present study, GLUT1 protein expression was observed in the EDC and gastrocnemius muscles of the English sparrow using immunohistochemistry (Fig. 3.2). However, immunostaining patterns were very different between EDC and gastrocnemius muscles. The EDC muscles showed immunostaining in areas associated with capillaries (Fig. 3.2b) whereas immunostaining of the gastrocnemius revealed a more heterogeneous pattern (Fig. 3.2c) consistent with the muscle being composed of different fiber types (Pette and Staron 1990; Torrella et al. 1998; Scott et al. 2001). Some gastrocnemius fibers immunostained more intensely than others and were surrounded by a larger number of capillaries consistent with an oxidative-type muscle fiber whereas others showed no significant immunostaining and very low capillary density consistent with glycolytic fibers. This immunostaining pattern was very similar to that observed in rat soleus muscle (Fig. 2d), a muscle known to be comprised of primarily oxidative fibers. Intense staining was seen in the rat soleus muscle in highly vascularized areas and appeared to stain within the muscle fibers as well. More studies are required to determine the fiber type composition of the avian muscles. The localization of GLUT1 to areas of abundant vascularization is consistent with GLUT1 localization to blood-tissue barriers (Uldry and Thorens, 2004; Shepherd and Kahn, 1999).

GLUT1 gene expression was observed in all sparrow tissues examined (Fig. 3.3a) and shares 88% gene sequence homology with chicken and 84% with human GLUT1 sequences (Fig. 3.5).

Using primers designed from the chicken genome sequence, a partial sequence of the sparrow GLUT3 gene was elucidated and its expression was found in all tissues examined (Fig. 3.3b). Moreover, the sparrow GLUT3 partial gene sequence shares 90% identity with chicken embryonic fibroblast and 75% with human GLUT3 (Fig. 3.6). As the CEF-GT3 cDNA expressed in rat fibroblasts encodes for a functional transporter (White et al., 1991), it is likely that the sparrow sequence does as well. It remains uncertain as to the localization of this protein. It is possible that the sparrow GLUT3 is localized mainly to nervous tissue as seen in rats and mice, but it is equally likely that it could be present in muscle tissue as observed in humans. Data for GLUT3 protein localization in sparrow skeletal muscle were equivocal (data not shown) as the C-terminus, against which antibodies are typically designed, varies greatly among GLUT3 proteins in mammals. An antibody for the avian protein sequence was not used as it is not commercially available. Our results from RT-PCR experiments are supportive of the lack of an adequate mammalian GLUT3 antibody to detect the avian protein as mammalian and sparrow GLUT3 share only 75% gene homology.

Concluding Remarks

In summary, our results demonstrate the lack of GLUT4 gene and protein expression in sparrow skeletal muscle. GLUT1 protein expression in this tissue was localized mainly to areas containing a high concentration of blood vessels (blood-tissue barriers). Gene expression was found in all tissues examined and was highly homologous to the chicken and human sequences. Expression of the GLUT3 transport protein could not be determined using antibodies to the mammalian protein as an avian antibody is not commercially available to date. However, gene expression of this transporter was identified in all tissues examined and shares 90% gene sequence homology with chicken embryonic fibroblast, and 75% homology with human, GLUT3 sequences. The minimal expression of glucose transport proteins in avian skeletal muscle supports the observed low levels of glucose transport by this tissue (Sweazea and Braun, 2005). What is unclear is the physiological basis for the maintenance of the high plasma glucose concentrations in avian plasma.

CHAPTER 4

PARTIAL CHARACTERIZATION OF OLEIC ACID UPTAKE INTO ENGLISH SPARROW SKELETAL MUSCLE

Abstract

Studies of prolonged flight have shown it to require large amounts of energy supplied mainly by free fatty acids (FFA). In the present study, the high levels of plasma ketone bodies found in sparrows (2.58mmol l^{-1}) is supportive of this observation. In addition, the effects of various pharmacological agents on oleic acid (OA) uptake by English sparrow *extensor digitorum communis* (EDC) muscles were examined. Initial studies demonstrated that radiolabeled OA uptake decreased in the presence of increasing unlabeled OA, suggesting that OA uptake is occurring by a facilitative transport process. To further characterize OA uptake, EDC muscles were incubated with either: insulin (2ng/ml), IGF-I (48ng/ml), AICAR (2mmol) or caffeine (5mmol). Insulin, but not IGF-I, significantly increased OA uptake by avian EDC ($P<0.01$). Caffeine and AICAR were ineffective at increasing avian OA uptake. DIDS (500 μmol), an inhibitor of protein-mediated transport, increased OA uptake by 60 minutes. Therefore the effects of other inhibitors of FFA uptake were examined. Phloretin (200μmol) also significantly ($P<0.05$) increased OA uptake at 60 minutes. However, a specific inhibitor of LCFA transport by fatty acid transporters (FAT/CD36), sulfo-*N*-succinimidyl oleate (SSO; 500μmol), significantly decreased OA uptake at 2.5 min. The effectiveness of SSO suggests that a FAT/CD36-like protein is expressed in avian tissues. As uptake of OA was not

completely blocked by SSO, and was increased by DIDS and phloretin, it is likely that other mechanisms for FFA movement across membranes may be present.

Introduction

Birds are exceptional models of the use of lipids to meet the demands of high basal metabolic rates and prolonged flight (Blem, 1990; Jenni-Eiermann et al., 2002). English sparrows, small passerine birds, have metabolic rates (2.46 ml O_2/g/h) that are much higher than non-passerine birds (Hulbert et al., 2002). Sparrows store 14% of their body weight as fat to survive the fasting energy requirement during the night (10.2-22.2 kcal). The major long chain fatty acid (LCFA) stored in English sparrows are palmitic (C16:0) and oleic acid (C18:1) (Blem, 1976). These LCFA provide significant amounts of energy in comparison to glucose: palmitate 9.30kcal/g; oleate 9.40kcal/g, whereas carbohydrates provide only 4.0kcal/g (Blem, 1990; Randall et al., 2002).

Although birds apparently use free fatty acids (FFA) to supply the energy demands of skeletal muscle to produce flight, it is not clear how these FFA are taken up by the muscle cells. As skeletal muscle cells do not have the capacity to synthesize FFA, these substrates have to be delivered to the cells by the circulation. In the blood, FFA bound to serum albumin dissociate rapidly making them available for cellular uptake (McArthur et al., 1999; Hajri and Abumrad, 2002). Once reaching the sarcolemma, there are at least two processes by which FFA can enter cells, passive diffusion and facilitated transport (McArthur et al., 1999; Hajri and Abumrad, 2002). Free fatty acid diffusion via a "flip-flop" mechanism through the plasma membrane occurs at a relatively fast rate of 1-10

molecules per second for palmitate and oleate. Transport of FFA by a carrier has a Km of 7nM and is thought to be the rate-limiting step of fatty acid metabolism in tissues (Hajri and Abumrad, 2002). In addition, a study by Richards et al. (2004) demonstrated that palmitate movement into rainbow trout sarcolemmal vesicles occurred by a saturable process sensitive to inhibition by chemical agents and competitively inhibited by other LCFA, suggestive of a facilitative process. There are a number of known FFA transport proteins in the cell membranes of a variety of tissues. The main players appear to be: fatty acid translocase (FAT/CD36), fatty acid transport protein (FATP), and plasma membrane fatty acid binding protein (FABPpm).

After crossing the plasma membrane, FFA enter the cytosol where they are bound by a 14kDa cytosolic fatty acid binding protein (FABPc). This protein acts as an intracellular sink for FFA as shown by cardiac muscle FABPc-null mice that display decreased FFA uptake (Schaap et al. 1999; Luiken et al., 2002a). Cytosolic FFA destined for beta-oxidation are chaperoned by FABPc to the mitochondria where they are oxidized to form ATP (Spriet, 2002).

The FABPc of Western sandpipers was found to comprise ~13.7% of pectoralis and 21.3% of cardiac muscle cytosolic proteins, the highest levels found in any vertebrate, and is highly (80-86%) homologous to human and trout sequences (Guglielmo et al., 1998). For comparison, FABPc levels in mammals are typically 2-5% of cytosolic proteins (McArthur et al., 1999).

According to Rasmussen et al. (2004), the mitochondrial respiratory chain capacity of pigeon pectoralis muscle is sufficient for maximal fatty acid and carbohydrate respiration.

However studies by Sweazea and Braun (2005) have shown that, in comparison to mammals, avian skeletal muscle does not take up glucose to an appreciable extent.

Most of the published studies have examined fatty acid distribution and costs of flight using whole animal models, mainly migratory birds. Therefore the purpose of the present study was to characterize *in vitro* skeletal muscle free fatty acid uptake in a non-migratory bird, the English sparrow (*Passer domesticus*) utilizing the same methods as for the glucose transport studies (Sweazea and Braun, 2005). Oleic acid was used as it is a major fatty acid stored by these birds. These studies were not designed to measure fatty acid metabolism, but rather, to study the uptake of oleic acid as a potential energy source for isolated avian skeletal muscles co-incubated in the presence of various pharmacological agents.

Materials and Methods

Experimental Animals and Tissue Preparation

On the morning of each experiment, English sparrows (*Passer domesticus*; body mass 24.1 ± 0.15g (mean ± SEM; n=92)) were captured by mist net at the University of Arizona Dairy Research Center (Tucson, AZ). Birds were anesthetized using 65mg/ml sodium pentobarbital (40 µl) and the *extensor digitorum communis* (EDC) muscles extracted. The EDC is a relatively small muscle (24.2 ± 0.23mg (mean ± SEM; n=184)) located between the radius and ulna. The well-defined tendons on either end of the

muscle allow for extraction with minimal damage. All animal protocols were approved by the University of Arizona Institutional Animal Care and Use Committee (IACUC).

Measurement of Ketone Bodies in Whole Arterial Blood

Prior to muscle extraction, arterial blood samples were collected into heparinized capillary tubes from anesthetized sparrows. These samples were analyzed for ketone bodies using PTS PANELS ketone test strips and read with a CardioChek P•A meter (Polymer Technology Systems, Indianapolis, IN). In studies by Guerci et al. (2003) the results obtained using this method strongly correlated with those derived using spectrophotometric methods.

Protocol for ALL Oleic Acid Uptake Studies.

Oleic acid (OA) uptake was examined by the *in vitro* incubation of muscles using a protocol adapted by Sweazea and Braun (2005) from Bonen et al. (1994). For all studies, FFA uptake was quantified using [9,10-^{3}H(N)]-oleic acid (PerkinElmer Life Sciences, Boston, MA). Excised EDC muscles were incubated in three successive temperature-controlled water baths (41°C, sparrow body temperature) all of which contained Krebs Henseleit buffer (KHB: 110mmol NaCl, 5mmol KCl, 2mmol NaH_2PO_4, 1mmol $MgSO_4$, 25mmol $NaHCO_3$, 1.8mmol $CaCl_2$; pH 7.45) as the background solution. Various amino acids (5mmol L-alanine, 10mmol Na-acetate, 1mmol Na-citrate, 1.5mmol Na-lactate, 1mmol Na-malate, 0.9mmol glycine) were added to the KHB solution. The incubation

solutions were continually gassed with 95%O_2:5%CO_2. To maintain an osmolality of 300mOsm/kg-H_2O, the total solute concentration for each solution was adjusted to 40mmol using mannitol.

The first incubation solution consisted of 1mmol OA and 39mmol mannitol to bring all muscles to the same physiological state (20 min). The second incubation, a 10-minute rinse to remove excess OA, contained only 40mmol mannitol. The final incubation solution (the test solution) was comprised of 15.2μCi/mmol [9,10-^{3}H(N)]-oleic acid (^{3}H-OA) and 0.8μCi/mmol [U-^{14}C]-mannitol (MP Biomedicals, Irvine, CA) with varying levels of unlabeled substrates to be described for each experiment. Unless otherwise indicated, final incubations were carried out for 60 minutes as maximal uptake of OA was observed at this time point as shown by control values in Figure 4.3. The muscles were subsequently rinsed in ice-cold KHB, clamp frozen in liquid nitrogen, and weighed (to 0.1mg). Muscles were then digested at 60˚C in scintillation vials containing 500μL of 0.5N NaOH for 2 hours. Ecolite(+) liquid scintillation cocktail (MP Biomedicals, Irvine, CA) was added to each vial and the homogenates were dark adapted for three hours prior to counting. Radiolabeled mannitol was used in the final incubation solution as it is not transported into skeletal muscle cells and can therefore be used to quantify the extracellular space. Using the specific activity of the isotope, the ^{3}H-OA counts were converted to moles ^{3}H-OA uptake per mg muscle (wet weight) from which the mannitol counts were subtracted to account for free OA in the extracellular space.

Competitive Uptake of Oleic Acid

To determine the facilitated nature of OA uptake, muscle pairs were placed in final incubation solutions containing incremental levels of unlabeled OA (0, 2.5, 5, 10, 20mmol) with the addition of ^{3}H-OA and [U-^{14}C]-mannitol. At concentrations above 1mmol, OA is relatively insoluble in the heated KHB solution. To facilitate dissolution, 0.3% chicken serum albumin, 0.8% ethanol, and 0.4% DMSO were added to the KHB solution.

The Effects of Pharmacological Agents on Oleic Acid Uptake

The left EDC muscle from each bird, the control, was incubated in 1mmol OA and 39mM mannitol in the final incubation. The contralateral muscle (experimental) received the same treatment as the control up to the final incubation at which point the experimental muscles were incubated in the presence of various pharmacological agents (caffeine, AICAR, insulin, IGF-1, DIDS, phloretin, SSO) known to affect different aspects of substrate uptake. Unless otherwise stated, all final incubations were carried out for 60 minutes as this time point represented maximal OA uptake (Refer to control data shown in Fig. 4.3) while minimizing muscle degeneration due to prolonged incubation.

Caffeine: Stimulation of calcium release from the sarcoplasmic reticulum, as occurs during muscle contractions, can be induced using caffeine, a ryanodine receptor agonist (Freymond et al., 2002). Caffeine (5mmol) was added to the final incubation solution, which also contained 1mmol OA and 34mmol mannitol (n=9 paired muscles).

AICAR: 5'-Aminoimidazole-4-carboxamide-1-b-D-ribofuranoside (AICAR) has been shown to increase FFA oxidation by mimicking AMP increases in muscle as occurs during contraction (Rasmussen and Wolfe, 1999; Kaushik et al., 2001). Fifteen paired experimental and control muscles were used for this study. The final experimental incubation solution was comprised of 2mmol AICAR (Toronto Research Chemicals, North York, Ontario), 1mmol OA and 37mmol mannitol.

Insulin: Insulin has been used to enhance palmitate transport in cardiac myocytes derived from rats (Coort et al., 2002). Chicken insulin (Gift from JP McMurtry) was used for these studies as sparrow insulin is not commercially available. Nine birds were used for this experiment (paired control and experimental muscles). Insulin (2ng/ml) was added to the final incubation solution along with 1mmol OA and 39mmol mannitol.

IGF-1: As the uptake of OA by mammalian skeletal muscle increases in response to insulin, the effects of IGF-1 on OA uptake were examined. IGF-1 mimics the effects of insulin by binding to, and activating, the insulin receptor (Poggi et al. 1979). Human IGF-1 (Sigma, St. Louis, MO) was used in our studies as it displays a high degree of sequence similarity with avian IGF-1, which is not commercially available (Ballard et al., 1990). The final incubation solution consisted of IGF-1 (48ng/ml), 1mmol OA and 39mmol mannitol. Five paired control and experimental muscles were used for this study.

DIDS: 4,4'-diisothiocyanostilbene-2,2'disulfonate (DIDS) has been shown to nonspecifically reduce protein-mediated transport of FFA (Abumrad et al., 1998; Coort et al., 2002; Richards et al., 2004). Five paired experimental and control muscles were used for this study with one pair incubated at each time point (0, 2.5, 5, 10, 60 minutes). The final incubation solution contained 500µM DIDS, 1mmol OA and 39mmol mannitol.

Phloretin: A non-selective inhibitor of protein-mediated transport, phloretin, has been used to inhibit FFA uptake in studies using mammalian tissues (Abumrad et al., 1998; Bonen et al., 1998, Luiken et al., 1999b; Coort et al., 2002; Richards et al., 2004). Phloretin (200µmol) was added to the final experimental incubation medium, which also contained 1mmol OA and 39mmol mannitol (n=15 paired control and experimental muscles).

Sulfo-N-succinimidyl Oleate (SSO): A LCFA (i.e. oleate) coupled to *N*-hydrosulfosuccinimide forms a sulfo-*N*-succinimidyl ester of OA. This agent has been used to inhibit palmitic acid and OA transport (Abumrad et al., 1998; Bonen et al., 1998; Coort et al., 2002). SSO is a lipophilic, albeit membrane impermeable, substrate that cannot cross-link with proteins. It is therefore specific to membrane bound FFA transporters and has been shown to covalently bind only to FAT/CD36 to inhibit LCFA uptake (Bonen et al., 2000; Coort et al., 2002). SSO was produced by the University of Arizona Chemical Synthesis Facility (Dr. Ronald Wysocki). Six paired experimental and control muscles were incubated for 2.5 minutes, and 7 muscle pairs for 20 minutes, in a final incubation medium consisting of 500µmol SSO, 1mmol OA and 39mmol mannitol.

Statistics

The quantity of OA taken up by EDC muscles was expressed as moles OA uptake per mg muscle (wet weight). The final data for each experiment are shown as means ± SEM. Student's T-test was used to determine the level of significance between control and experimental values. $P<0.05$ was the accepted level of significance.

Results

TABLE 4.1. Comparison of Ketone Levels Among Various Avian Species.

			β-hydroxybutyrate	
Species	Sample	*N*	mg $100ml^{-1}$	mmol l^{-1}
English Sparrow (rest)‡	Whole Blood	22	26.9 ± 2.49*	2.58
Red Knot (flying)‡‡	Whole Blood	35	13.8	1.33 ± 0.79§
European Robin (rest)‡	Plasma	25	28.4	2.73 ± 1.31§§
Pied Flycatcher (rest)‡	Plasma	18	38.2	3.67 ± 1.16§§
Garden Warbler (rest)‡	Plasma	16	25.6	2.46 ± 1.33§§
Blackcap (rest)‡	Plasma	9	32.3	3.10 ± 1.16§§

*Present Study, Mean ± SEM.
§Jenni-Eiermann, 2002.
§§Jenni-Eiermann, 2001.
‡Passeriformes; ‡‡ Charadriiformes

Competitive Uptake of Oleic Acid

The uptake of ^{3}H-OA decreased in the presence of increasing concentrations of unlabeled OA and was almost completely inhibited with 20mmol OA. This curve demonstrates the competitive nature of ^{3}H-OA uptake indicating that a LCFA facilitative transport protein(s) may be present in the EDC muscle (Fig. 4.1).

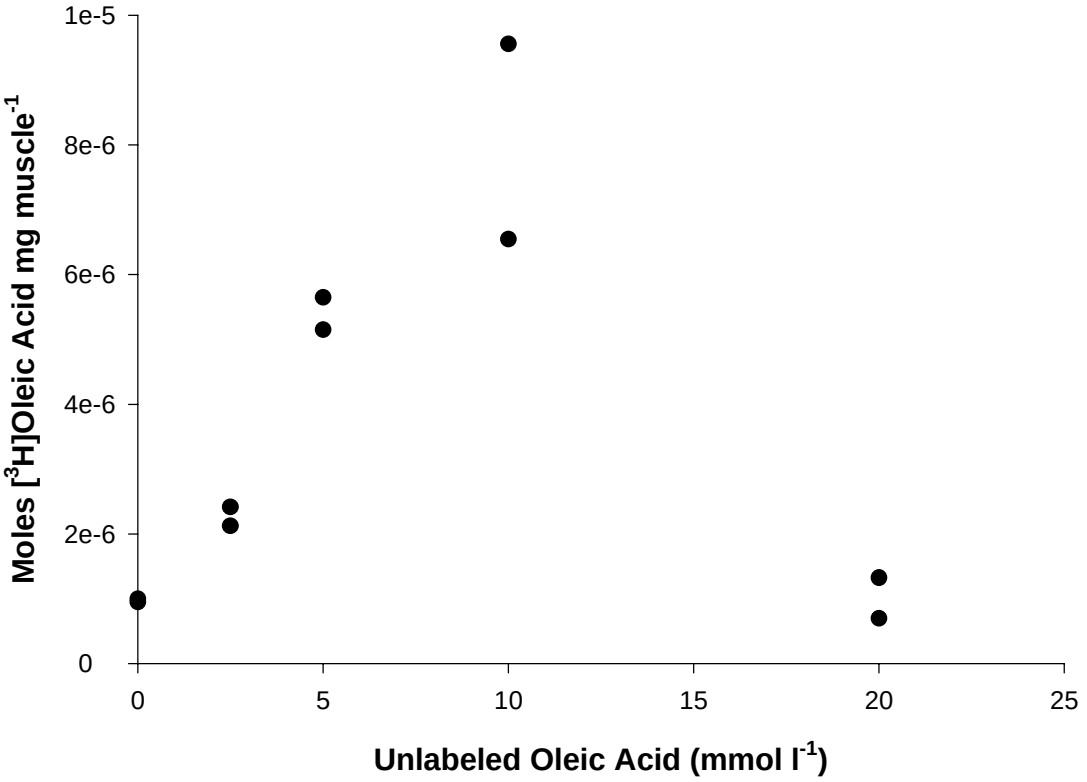


FIGURE 4.1. Competitive Uptake of Radiolabeled Oleic Acid (OA) in the Presence of Unlabeled OA by *Extensor Digitorum Communis* Muscles Incubated *In Vitro*.

Data (mean ± SEM) expressed as moles [9,10-^{3}H(N)]-OA uptake per mg muscle (wet weight). Two muscles were incubated at each concentration. Labeled OA uptake decreased in the presence of incremental concentrations of unlabeled OA (0, 2.5, 5, 10, 20mmol) in the final incubation (60min). Uptake of labeled OA was maximally inhibited by 20mmol unlabeled OA.

The Effects of Pharmacological Agents on Oleic Acid Uptake

Caffeine

The effects of muscle contractions can be mimicked *in vitro* using caffeine, a ryanodine receptor agonist that stimulates the release of calcium from the sarcoplasmic reticulum. In the present study, the addition of caffeine (5mmol) to the final incubation medium had no effect ($P>0.05$) on ^{3}H-OA uptake by the nine paired EDC muscles (Fig. 4.2; Control: 2.49 ± 0.38; Experimental: 2.12 ± 0.37 μmol mg^{-1}). Increased cytoplasmic calcium levels do not appear to affect FFA uptake by avian skeletal muscle at 60 min of incubation.

AICAR

AICAR (5'-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside) increases substrate uptake into skeletal muscle by mimicking increased intramuscular AMP (as ZMP (5'-aminoimidazole-4-carboxamide ribonucleotide)). Fifteen paired muscles were used for this study. Experimental muscles were incubated in the presence of 2mmol AICAR. Uptake of ^{3}H-OA did not differ significantly ($P>0.05$) between control (2.97 ± 0.30 μmol mg^{-1}) and experimental (2.89 ± 0.27 μmol mg^{-1}) muscles. Therefore, AICAR does not significantly increase OA uptake by *in vitro* avian skeletal muscle at 60 min incubation (Fig. 4.2).

Insulin

The addition of chicken insulin (2ng/ml) to the final incubation medium significantly ($P<0.01$) increased ^{3}H-OA uptake by EDC muscles over control values at 60 minutes of incubation time (Fig. 4.2). Values for nine paired control and experimental muscles were 1.92 ± 0.23 and 2.81 ± 0.43 μmol mg^{-1}, respectively. These data indicate sparrow EDC muscle OA uptake is sensitive to insulin.

IGF-1

Insulin like growth factor isoform-1 (IGF-1) has been shown to activate insulin-sensitive pathways in skeletal muscle by binding to, and activating, the insulin receptor. To test the effects of IGF-1 on ^{3}H-OA uptake in comparison to insulin, this agent was added to the final incubation solution (48ng/ml). The results (Fig. 4.2) demonstrate no significant ($P>0.05$) increase in ^{3}H-OA uptake in the presence of IGF-1 at 60 min (Control: 2.15 ± 0.36; Experimental: 2.37 ± 0.39 μmol mg^{-1}; n=5 paired muscles).

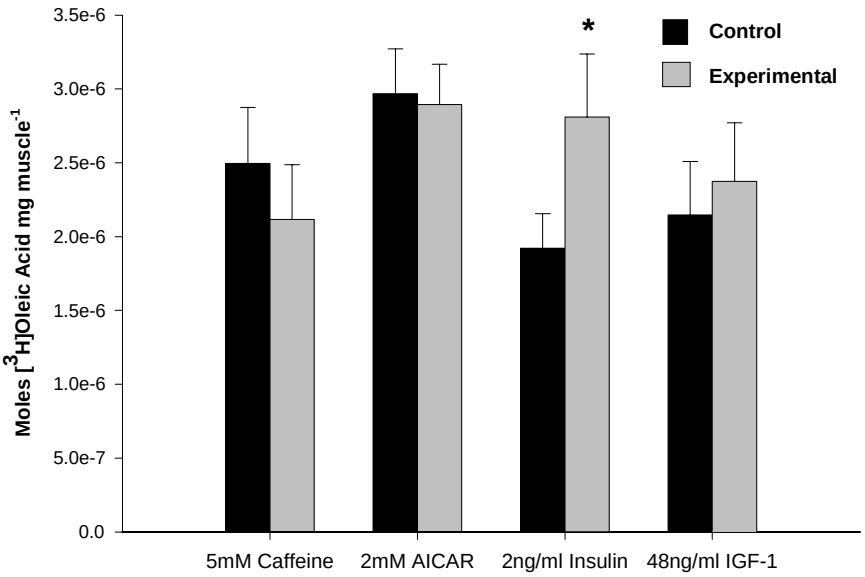


FIGURE 4.2. Effects of Pharmacological Agents on Oleic Acid (OA) Uptake by Paired EDC Muscles.

No significant difference exists between control muscles (black bars). Paired contralateral muscles (gray bars) were exposed to various agents in the final incubation solution (60min). Caffeine (5mmol), a ryanodine receptor agonist, had no effect on OA uptake (n=9). The addition of AICAR (2mmol), an activator of AMPK, did not increase OA uptake (*n*=15). Chicken insulin (2ng/ml) significantly increased OA uptake (*n*=9). Addition of IGF-1 (48ng/ml) to the final incubation produced no significant effects on OA uptake (*n*=5). Values (mean ± SEM) are expressed as moles [9,10-^{3}H(N)]-OA uptake per mg muscle (wet weight). $^{*}P<0.008$

DIDS

DIDS (4,4'-diisothiocyanostilbene-2,2' disulfonate) is an agent used to inhibit substrate transport. However, ^{3}H-OA uptake appears to increase in the presence of DIDS at 60 minutes of incubation. Consequently, the use of DIDS to decrease free fatty acid uptake was abandoned and other inhibitors of fatty acid uptake were examined. As only two muscles were run per time point for this study, no statistics are available on this data (Fig. 4.3).

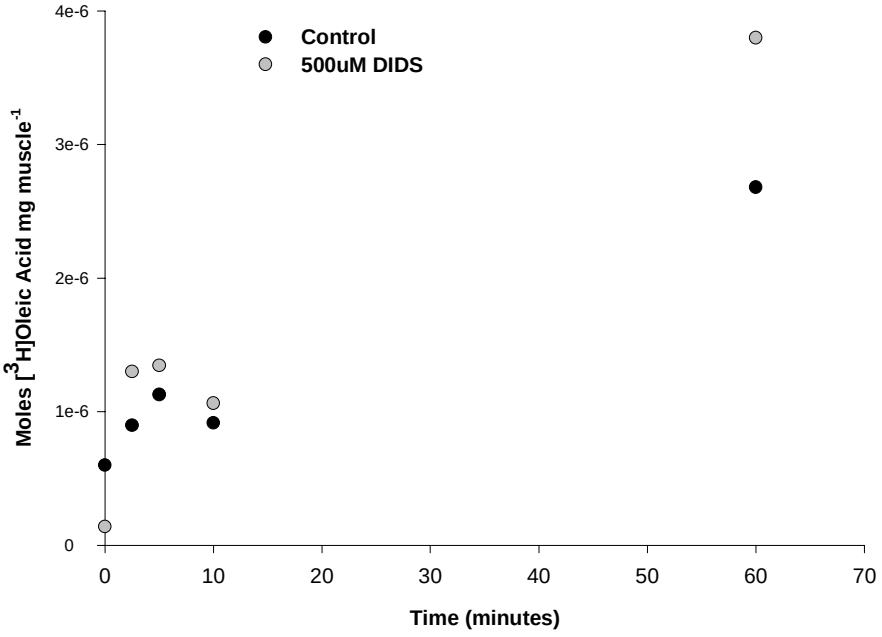


FIGURE 4.3. Time Course (0, 2.5, 5, 10, 60 min) of Oleic Acid Uptake in the Presence of a Non-Specific Inhibitor of Substrate Transport.

This inhibitor, DIDS (500µM), was added to the final incubation solution. The uptake of oleic acid was not inhibited by DIDS, but rather, there was a tendency for uptake to increase in the experimental muscles. Statistical data is unavailable as only one set of muscles was run at each time point. Values are expressed as moles [9,10-^{3}H(N)]-oleic acid uptake per mg muscle (wet weight).

Phloretin

Phloretin is an inhibitor of protein-mediated transporters. As shown in figure 4.4, at a concentration of 200μmol, this agent significantly ($P<0.024$) increased ^{3}H-OA uptake by the fifteen experimental EDC muscles at 60 minutes of incubation (2.24 ± 0.28 μmol mg^{-1}) in comparison to the paired control muscles (1.39 ± 0.15 μmol mg^{-1}).

SSO

SSO (sulfo-*N*-succinimidyl oleate) inhibits the transport of LCFA across muscle membranes by specifically blocking FAT/CD36. The final incubation solution contained 500μmol SSO. Six muscles were incubated in this solution for 2.5 minutes and seven were incubated for 20 minutes (Fig. 4.4). SSO significantly ($P<0.005$) decreased ^{3}H-OA uptake at 2.5 minutes of incubation (0.311 ± 0.09 μmol mg^{-1}) in comparison to the paired control values (0.69 ± 0.054 μmol mg^{-1}).

The experiments presented above, with the exception of DIDS and the competitive OA, were conducted using paired experimental and control muscles from each bird. The control values appeared to vary somewhat, although not significantly ($P>0.05$), between experiments using different pharmacological agents. This may be due to the various pathways by which FFA can move across cell membranes, i.e. diffusion and facilitated transport. This movement of FFA is facilitated by a diverse number of proteins: FAT/CD36, FATP, FABPpm, and FABPc, all of which may be contributing to FFA uptake in avian skeletal muscle.

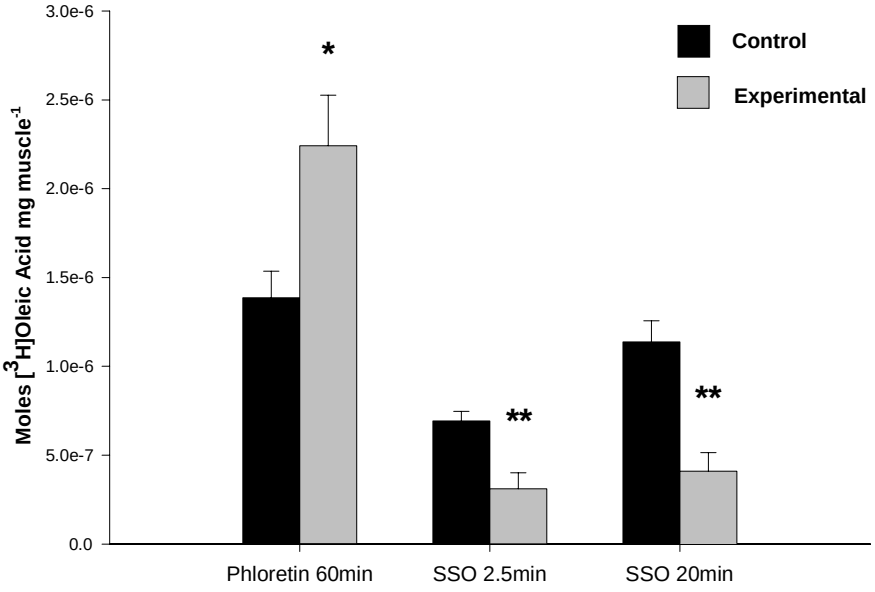


FIGURE 4.4. Effects of Inhibitory Pharmacological Agents on Oleic Acid (OA) Uptake by Paired EDC Muscles at Varying Time Points.

The uptake of OA by paired control (black bars) and experimental (gray bars) muscles was compared. Phloretin (200µM), a nonspecific inhibitor of transport proteins was added to the final incubation solution (60 min) and, like DIDS (Fig 3), significantly increased OA uptake by 38% (*n*=15) compared to its control. SSO, an ester of OA used to inhibit LCFA uptake by FAT/CD36, effectively decreased OA uptake by 55% at 2.5 minutes and by 64% at 20 minutes of incubation (*n*=7 each) compared to the paired controls. Data (mean ± SEM) expressed as moles [9,10-^{3}H(N)]-OA uptake per mg muscle (wet weight). $^{*}P<0.05$; $^{**}P<0.005$

Discussion

To our knowledge, the current study is the only one in which FFA utilization has been examined in isolated skeletal muscle from a non-migratory bird. This is significant, as it has been demonstrated that birds predominantly utilize FFA as a metabolic fuel. Data from studies of migratory and birds exercised in a laboratory support this point (Jenni-Eiermann et al., 2002). As a result of this metabolism, the major ketone body in avian plasma is β-hydroxybutyrate (Klasing, 1998). Increased levels of β-hydroxybutyrate have been shown to decrease glucose utilization thereby sparing protein and carbohydrates, especially during fasting (Robinson and Williamson, 1980). In the present study, the level of β-hydroxybutyrate in sparrow whole blood was 26.9 ± 2.49 mg $100ml^{-1}$ suggesting a high level of FFA metabolism. This value for English sparrows is more than fifty times higher than normal human whole blood concentrations (0.5mg $100ml^{-1}$; Diem, 1962) but similar to values reported for other birds (Table 4.1).

Data from the present study demonstrate that FFA uptake by avian skeletal muscle occurs via a facilitative process as ^{3}H-OA accumulation by muscle cells is decreased by SSO and competitively inhibited in the presence of incremental unlabeled OA (Figs. 4.1, 4.4). Free fatty acid utilization by muscles begins with FFA movement across cell membranes via a variety of mechanisms involving diffusion and transport proteins. Among the transport proteins is fatty acid protein (FABPpm) which has been detected in the plasma membrane of a wide variety of tissues (Stremmel et al., 1985; Schaffer and Lodish, 1994; Coort et al., 2002; Hajri and Abumrad, 2002). Intracellularly, a cytosolic

FABP (FABPc) binds to FFA thereby acting as a sink for FFA uptake into tissues (Schaap et al., 1999; Luiken et al., 2002a).

Chemical agents are commonly used to mimic *in vitro* muscle contraction. Caffeine, a ryanodine receptor agonist, stimulates the release of calcium from the sarcoplasmic reticulum. In mammals, increased intramyocellular calcium enhances substrate transport and utilization. It is conceivable that increased cytosolic calcium would enhance FFA uptake as occurs during prolonged flight. However, in the present study, caffeine was shown to have no effect on *in vitro* muscle ^{3}H-OA uptake at 60 minutes of incubation (Fig. 4.2). This suggests 3H-OA uptake is not increased in response to an increase in cytosolic calcium and/or translocation of FAT/CD36 is not likely to occur via a calcium-induced mechanism as proposed for mammals (Spriet, 2002).

Muscle contraction results in an increase in intracellular AMP levels which, in turn, phosphorylates AMP-activated protein kinase (AMPK). Through a cascade of intracellular events beginning with AMPK, the rate-limiting mitochondrial enzyme for FFA oxidation, carnitine palmitoyl transferase (CPT-I), is activated (Winder et al., 1990; Saggerson et al., 1992). AICAR is commonly used to activate AMPK as it mimics increased cytosolic AMP levels and has been demonstrated to increase FFA oxidation by mammalian tissues (Rasmussen and Wolfe, 1999; Kaushik et al., 2001). The results of the present study (Fig. 4.2) indicate that avian skeletal muscle does not respond to AICAR with increased ^{3}H-OA uptake at 60 minutes of incubation. This suggests that translocation of FAT/CD36 may not occur by an AMPK-dependent mechanism and/or that increased FFA oxidation and transport are not coupled (Spriet, 2002).

A separate pathway by which FAT/CD36 translocation to the plasma membrane can be enhanced is via the insulin-signaling cascade. This is thought to occur via a mechanism involving phosphoinositide 3-kinase (PI3K) (Dyck et al., 2001; Luiken et al., 2002b). Insulin has been shown to increase palmitate transport into cardiac myocytes derived from fasted rats (Coort et al., 2002) as well as in rested and contracting rat soleus muscles (Dyck et al., 2001). Moreover, in perfused rat hindlimb muscles, insulin enhanced palmitate uptake into skeletal muscle and its incorporation into phospholipids, triacylglycerols, and diacylglycerols while reducing its oxidation. Of note, the effects of insulin on inhibition of FFA oxidation are much less than its effects on esterification. Despite previous findings that birds are resistant to insulin-stimulated increases in glucose transport (Sweazea and Braun, 2005) results from the present study demonstrate insulin is effective at significantly ($P<0.01$, Fig. 4.2) enhancing the uptake of ^{3}H-OA into isolated EDC muscles by 32% at 60 minutes of incubation. However, these tissues were not responsive to stimulation by IGF-1, which typically increases glucose transport in mammalian tissues via the insulin receptor signaling pathway as well as its own receptor (Fig. 4.2). translocase (FAT/CD36), an 88kDa integral glycoprotein that is present in muscle and other tissues (Abumrad et al., 1984; Hajri and Abumrad, 2002). There is evidence that this protein translocates to the sarcolemma in response to exercise and is thought to occur by a calcium or AMP dependent mechanism (Spriet, 2002). In addition, insulin has been shown to induce translocation of FAT/CD36 via a separate pathway (Luiken et al., 2002b). Other fatty acid transporters, such as fatty acid transport protein (FATP) and plasma membrane fatty acid binding Phloretin and DIDS, both commonly

used to inhibit substrate transport in mammalian tissues, increased ^{3}H-OA uptake by avian skeletal muscle (Figs 4.3, 4.4). Phloretin increased ^{3}H-OA uptake by 38% ($P<0.05$, Fig. 4.4). As these agents are inhibitors of protein-mediated transport activity, the increased uptake of ^{3}H-OA may occur by increased diffusion of fatty acids. The EDC muscle has been shown to consist primarily of fast oxidative glycolytic fibers (Braun, unpublished) and oxidative fiber types express large amounts of FABPc compared to glycolytic fibers (Rasmussen and Wolfe, 1999). Birds have been shown to have the highest expression of FABPc among vertebrates (Guglielmo et al., 1998). As FABPc has a high affinity for LCFA, these intracellular binding proteins may create a sink for oleic acid uptake thereby enhancing diffusion. Alternatively, it is possible that phloretin and DIDS block specific transport proteins or diffusion processes leaving the others unchanged.

Studies by Coort et al. (2002) have shown that SSO specifically inhibits FAT/CD36 without eliciting an effect on FATP or FABPpm. Expression of the different FFA transport proteins varies among tissues such that the effectiveness of SSO also varies (Bonen et al., 1998). In addition to blocking FAT/CD36 fatty acid uptake at the plasma membrane, SSO inhibits FAT/CD36 translocation thereby effectively decreasing uptake of fatty acids in response to stimulation (Bonen et al., 2000). In the present study, SSO significantly decreased ^{3}H-OA transport although complete inhibition was not observed (Fig. 4.4). At 2.5 minutes, SSO decreased ^{3}H-OA transport by 55% ($P<0.005$) compared to control values, and at 20 minutes, transport was decreased further by 64% ($P<0.005$). These results suggest birds express FAT/CD36, which transports a significant amount of

FFA across the sarcolemma. Birds more than likely express other FFA facilitative proteins, such as FATP or FABPpm, as FFA uptake was not completely inhibited in the presence of SSO. Our results are comparable to those of Luiken et al. (1999b) whose data showed 40-50% inhibition of palmitate uptake in rat skeletal muscle membrane vesicles with SSO. Again, as birds express the highest levels of FABPc among vertebrates, it is likely that this protein acts as an intracellular sink for FFA uptake. It is also probable that birds demonstrate variations in the relative concentrations of these plasma membrane and intracellular proteins, which would support the observable slight, but not significant, variations in control values (Fig. 4.2).

The protocols used in this study are very similar to studies that have been conducted by Turcotte et al. (1991) in which male Wistar rats were perfused with [1-^{14}C]-palmitate and blood was sampled at 10, 25, and 40 minutes post-infusion to study the uptake of palmitate from the blood into hindlimb muscles. In addition, Fürnsinn et al. (2000) incubated soleus muscle strips in [U-^{14}C]-palmitate for 60 minutes prior to extraction for analyses. Furthermore, Górecka et al. (2001) used male Wistar rat soleus muscle strips incubated in [1-^{14}C]-palmitate for 20 minutes prior to analysis.

Although the above cited studies were designed to measure fatty acid metabolism, they are demonstrative of prolonged incubations in a medium containing radiolabeled FFA. The presumption is that FFA are not completely metabolized by 60 minutes of incubation and it is therefore possible to gauge FFA uptake even at this prolonged incubation period. Undoubtedly, a portion of oleic acid is metabolized over this time course, but our studies allowed for the examination of the effects of pharmacological

agents on [^{3}H]OA uptake while exposed to [^{3}H]OA. As muscles were continuously bathed in an incubation medium containing [^{3}H]OA, these data represent the amount of uptake occurring at a specific time point, 60 minutes, which is sufficient time for the pharmacological agents to take effect. The agents used have different half-lives and act at different rates such that the protocol used in these studies allowed for comparison of the effects of the various agents over the same time frame. Of note, the uptake of [^{3}H]OA continued to be competitively inhibited by unlabeled OA at 60 minutes of incubation. Furthermore, studies using inhibitors of transport proteins were carried out over shorter time periods as these agents block the facilitated transport of OA across cell membranes and therefore do not require extended incubation times to take an effect which is in contrast to the other agents used, that act through various intracellular signaling pathways necessitating longer incubation periods. The addition of the non-metabolizable sugar, mannitol, to our studies allowed for the quantification of the extracellular space and was representative of the amount of OA not taken up by cells.

Concluding Remarks

In summary, birds have high levels of plasma ketone bodies suggestive of a high rate of FFA utilization. These FFA are taken up by avian skeletal muscle via a facilitative process as ^{3}H-OA accumulation by muscle cells is decreased by SSO and competitively inhibited in the presence of increasing unlabeled OA. Insulin enhances ^{3}H-OA transport potentially through activation of a PI3K-dependent pathway. Calcium and AMPK do not appear to affect FFA uptake by avian skeletal muscle, as caffeine and AICAR had no effect on OA uptake. Two commonly used inhibitors of protein-mediated transport were shown to increase ^{3}H-OA transfer into cells suggesting that FABPc may act as a sink for FFA diffusion and/or that other transport proteins may be resistant to inhibition by these agents. SSO, a specific inhibitor of FAT/CD36, significantly decreased ^{3}H-OA uptake indicating avian skeletal muscle contains a significant amount of FAT/CD36-like protein.

CHAPTER 5

RECIPROCAL INHIBITION OF *IN VITRO* SUBSTRATE MOVEMENT INTO AVIAN SKELETAL MUSCLE

Abstract

Birds display plasma glucose and ketone levels that are much higher than humans, and therefore, may offer a model in which to examine the effects of high plasma glucose and free fatty acid (FFA) levels on substrate preference. In the present study, uptake of radiolabeled oleic acid (OA; C18:1) and glucose by the extensor digitorum communis (EDC) muscle, isolated from the forewing of English sparrows, were examined. In dose-response studies, unlabeled glucose and OA (20mM each) inhibited the uptake of their respective radiolabeled counterparts. To examine the effects of glucose on OA uptake, muscles were incubated for 60 min in KHB buffer containing 20mM glucose with the addition of radiolabeled OA. This level of glucose significantly decreased radiolabeled OA uptake 36% ($P<0.02$). Using the same methodology, 20mM OA significantly decreased labeled glucose transport 49% ($P<0.00005$). Comparing control values for glucose (9.52e-7±4.0e-8) and OA (2.20e-6±2.9e-7) uptake, it is evident that OA is preferentially taken up by avian muscle (data in M substrate/mg muscle ± SEM). As FFA provide a greater amount of energy (146 ATP/OA) than carbohydrates (36 ATP/glucose), avian muscle utilizes FFA as an efficient energy source. It is likely that the high levels of FFA uptake by avian skeletal muscle induce the notable resistance to insulin mediated glucose uptake.

Introduction

Activity of vertebrates is supported by energy transduction derived from two main classes of metabolic substrates: carbohydrates and lipids, with protein (amino acids) playing a relatively minor role unless the others have been depleted (Randle, 1998). Birds utilize predominantly free fatty acids (FFA) to sustain flight while maintaining very high plasma glucose levels (Jenni-Eiermann et al., 2002). In addition, birds are resistant to the plasma glucose lowering effects of insulin, and other pharmacological compounds used to lower glucose levels in mammals (Sweazea and Braun, 2005).

In general, the etiology of insulin resistance remains to be determined; i.e. whether it involves glucose and/or FFA mediated processes. The uptake of fatty acids and glucose by muscle occurs by similar mechanisms involving the redistribution of transporters to the plasma membrane in response to stimuli (Bonen et al. 2000). Studies have shown that intramuscular lipid content correlates with insulin resistance suggesting that FFA play a role in the development of resistance to the glucose lowering effects of insulin (McGarry, 2002).

In the presence of basal levels of insulin, FFA have no effect on the rate of glycogen synthesis in rat isolated soleus muscle strips (Thompson et al., 2000). However, FFA decreased both the basal and insulin-stimulated rates of glucose transport and phosphorylation in these samples. Activation of insulin signaling pathway intermediates (protein kinase B (PKB)/Akt) were also reduced in the presence of FFA. These findings demonstrate that FFA directly induce *in vitro* skeletal muscle insulin resistance (Thompson et al., 2000). In whole body studies, elevated plasma FFA were shown to

decrease glucose disposal by 46%, and skeletal muscle glycogen synthesis by 50%, in humans. Glucose-6-phosphate was also decreased (by 25% at 45 minutes after FFA infusion) in the presence of increased plasma FFA suggesting that reduced glucose transport and/or phosphorylation may be the cause of the lower levels of glycogen synthesis. In addition, these effects of FFA were observed in the presence of both low (fasting) and high plasma insulin levels (Roden, 2004).

In mammals, increases in plasma FFA result in decreased insulin-mediated tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) and decreased IRS-1-associated phosphoinositide 3-kinase (PI3K) leading to reduced translocation of glucose transporters in response to insulin (Roden, 2004). A similar mechanism of FFA inhibition of glucose uptake may be occuring in birds and causing the observable hyperglycemia and insulin resistance. Plasma glucose levels in the English sparrow (refer to Table 5.1) are more than twice the level of nondiabetic humans (80-100mg/dl) whereas the uptake of glucose by avian skeletal muscle is nominal (Sweazea and Braun, 2005). In addition, glycogen levels in English sparrow skeletal muscle (Table 5.1) are well below normal levels observed in rat soleus muscles (17.5nM/mg wet wt) (Thompson et al., 2000).

Birds express intramuscular fatty acid binding proteins ($FABP_c$) at higher concentrations than other vertebrates (Guglielmo et al., 1998). Typical levels in mammalian tissues active in FFA metabolism are 2-5% of cytosolic proteins whereas concentrations in Western sandpipers, a migratory bird, are 13.7% of pectoralis and 21.3% of cardiac muscle cytosolic proteins (McArthur et al., 1999; Guglielmo et al., 1998, 2002). These high levels of FABPc enhance FFA uptake by acting as a

cytoplasmic sink for FFA (McArthur et al., 1999) as well as cytoplasmic FFA carriers to increase the mobility, and hence the utilization, of FFA within cells (Glatz et al., 2003). This is supported by the observation that FABPc increased from 6% of cytosolic protein in nonmigrating Western sandpipers to 10% during migration suggestive that birds increase FFA uptake from the circulation as opposed to increasing rates of FFA oxidation (Guglielmo et al., 2002). In addition, FABPc levels may be correlated with muscle fatty acid oxidation capacity (Haunerland et al., 1992; Luiken et al., 1999a; Glatz et al., 2003).

The levels of ketone bodies in birds (Table 5.1) are ~10x the level in nondiabetic humans (0.5mg $100ml^{-1}$; Diem, 1962). Sustained activity, such as flight, utilizes predominantly FFA which is supportive of the high observed ketone levels in birds as they arise as byproducts of FFA metabolism (Blem, 1976). The large capacity of avian skeletal muscle to take up FFA from the circulation may be the cause of the previously reported minimal skeletal muscle basal and insulin-induced glucose uptake (Sweazea and Braun, 2005). The present study addresses the hypothesis that elevated FFA contribute to this nominal *in vitro* skeletal muscle glucose uptake. The use of isolated skeletal muscles allows for direct measurement of the reciprocal effects of FFA and glucose uptake without the confounding factors of whole body metabolism. We suspect that FFA utilization by avian skeletal muscle renders the tissues resistant to insulin-mediated glucose uptake as well as decreasing basal levels of uptake.

TABLE 5.1. Physiological Data for English Sparrows.

Parameters	**Baseline Data**
Body Mass (g)	24.1 ± 0.15, (92)[a, b]
EDC Muscle Mass (mg)	24.2 ± 0.23, (184)[a, b]
Whole Blood Ketones (mg dl^{-1})	26.9 ± 2.49 (2.58 mmol l^{-1}), (22)[a, b]
Whole Blood Glucose (mg dl^{-1})	265.9 ± 53.5, (18)[a, c]
EDC Muscle Glycogen (nM mg wet $mass^{-1}$)	9.1 ± 4.11,(12)[a, c]

[a]Mean ± SEM, (*n*).
[b] Sweazea and Braun, (unpublished observations)
[c] Sweazea and Braun, (2005)

Materials and Methods

Experimental Animal and Tissue Preparation

English sparrows (*Passer domesticus*) were captured by mist net on the morning of each experiment at the University of Arizona Dairy Research Center. Birds were anesthetized with 40 µL of sodium pentobarbital (65 mg/ml) and the extensor digitorum communis (EDC) muscle extracted. The EDC is a small muscle (~23 mg) between the radius and ulna that has well defined tendons on either end allowing it to be dissected free without damaging the muscle. All animal protocols have been approved by the University of Arizona Institutional Animal Care and Use Committee.

Basic Protocol for All Transport Studies:

Radiolabeled glucose and mannitol were purchased from MP Biomedicals (Irvine, CA, USA) whereas radiolabeled oleic acid was purchased from PerkinElmer Life Sciences (Boston, MA, USA).

For all transport studies, the *in vitro* incubations were performed using a procedure as modified by Sweazea and Braun (2005) from Bonen et al. (1994). The uptake of glucose was quantified using [1,2-^{3}H]-2-deoxy-D-glucose ([^{3}H]2DG) and the uptake of fatty acids was quantified using [9,10-^{3}H(N)]-oleic acid ([^{3}H]OA). Oleic acid was used for these studies as it is a major fatty acid stored by English sparrows (Blem, 1976). Excised EDC muscles were incubated in two successive temperature controlled water baths (41˚C, sparrow body temperature; pH 7.45) all of which had Krebs Henseleit Buffer (0.11M NaCl, 5mM KCl, 2mM NaH_2PO_4, 1mM $MgSO_4$, 25mM $NaHCO_3$, 1.8mM $CaCl_2$) as the background solution. The KHB used was the standard buffer solution to

which was added several amino acids (5mM L-alanine, 10mM Na-acetate, 1mM Na-citrate, 1.5mM Na-lactate, 1mM Na-malate and 0.9mM glycine). The total solute concentration for each solution was adjusted to 40mM using mannitol as this concentration maintains an osmolality of approximately 300mOsm/kg-H_2O. Pre- and final incubation solutions contained identical concentrations of unlabeled substrates, the difference being the addition of radioisotopes to the final incubation solution. The pre-incubation step was carried out for 20 minutes and the final incubation lasted 60 minutes as this time point represented maximal uptake for each substrate. The incubation solutions will be described for each experiment. After the final incubation, the individual muscles were rinsed in ice-cold 2mM phloridzin (for glucose) or KHB (for oleic acid), clamp frozen in liquid nitrogen, and weighed (to 0.1 mg). The muscles were digested by 500 µL of 0.5N NaOH in liquid scintillation vials and placed in a 60˚C water bath for two hours. To account for background emission from NaOH, a 500µL aliquot of 0.5N NaOH was counted. Scintillation cocktail (EcoLite®) was added to each vial and the samples were dark-adapted for three hours prior to counting. Radiolabeled mannitol was used in the final incubation, as it is not transported and can therefore be used to quantify the extracellular space. Using the specific activity of the isotope, the oleic acid and glucose counts were converted to moles of substrate uptake/mg muscle from which the labeled mannitol counts were subtracted to account for free oleic acid in the extracellular space.

Competitive Uptake of Glucose:

The pre- and final incubation solutions for the experimental muscles contained 20mM D-glucose in addition to 20mM unlabeled mannitol in KHB solution. In addition to the unlabeled substrates, the final incubation had 300μCi/mM [^{3}H]2DG and 0.8μCi/mM [U-^{14}C]-mannitol. Five paired muscles were used for this experiment with control muscles exposed to 1mM glucose and 39mM mannitol in addition to the radioisotopes in the final incubation.

Competitive Uptake of Fatty Acids:

The control muscles (*n*=7) were incubated in 1mM oleic acid and 39mM mannitol. The pre- and final incubation solutions for the experimental muscles consisted of 20mM oleic acid and 20mM mannitol in KHB buffer. Radiolabeled oleic acid (15.2μCi/mM [^{3}H]OA) was added to the final incubation along with 0.8μCi/mM [U-^{14}C] mannitol.

Inhibition of Fatty Acid Uptake by Glucose:

Experimental muscles were incubated in pre- and final incubation solutions containing 20mM D-glucose and 20mM Mannitol along with 15.2μCi/mM [^{3}H]OA and 0.8μCi/mM [U-^{14}C]-mannitol. The uptake of labeled oleic acid by nine paired muscles was measured. The control muscles were exposed to 1mM glucose, 1mM oleic acid, 38mM mannitol with the addition of radioisotopes.

Inhibition of Glucose Uptake by Fatty Acids:

The uptake of glucose was measured in experimental muscles using pre- and final incubation solutions consisting of increasing concentrations of unlabeled oleic acid (1, 5, 10, 20mM) and a quantity of mannitol sufficient to bring the total solute concentration to 40mM. Seven muscles were incubated at each oleic acid concentration. In addition to the unlabeled substrates, the final incubation solution had 300μCi/mM [^{3}H]2DG and 0.8μCi/mM [U-^{14}C]-mannitol. The uptake of labeled glucose was measured after a 60-minute incubation. Control muscles (*n*=33) were incubated in 1mM glucose and 39mM mannitol for the pre and final incubations.

Statistics

The uptake of substrates after a 60-minute incubation were determined and averaged. Data are expressed as moles substrate uptake per mg muscle (wet weight) mean ± SEM. Student's T-test was used to determine the level of significance between control and experimental values. Significance was determined as $P<0.05$.

Results

Competitive Uptake of Glucose:

Unlabeled glucose (20mM) in the pre- and final incubations was effective at inhibiting the uptake of [^{3}H]2DG by 38% ($P<0.001$). Control values were: 8.4e-7 ± 2.92e-8 mol/mg and experimental values 5.2e-7 ± 3.45e-8 mol/mg (Fig. 5.1).

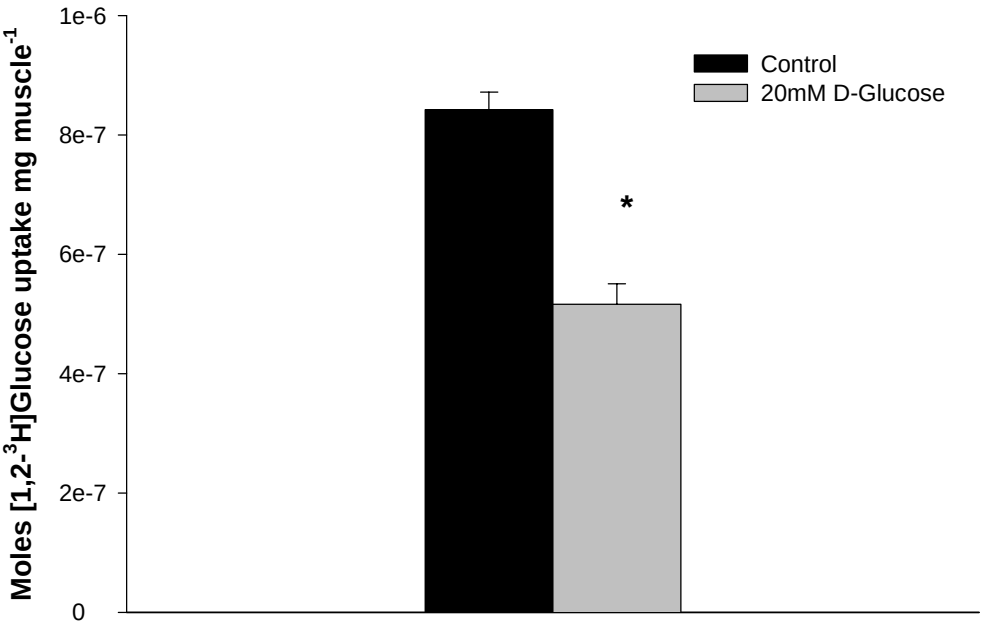


FIGURE 5.1. Competitive Uptake of [^{3}H]2DG in the Presence of 20mM D-Glucose.

D-glucose (20mM) in the pre- and final incubations inhibited the uptake of [^{3}H]2DG by 38% by 60 minutes of incubation. Data are expressed as the mean of Moles [^{3}H]2DG uptake per mg muscle (wet weight) ± SEM (*n*=5 paired muscles). *P<0.001

Competitive Uptake of Oleic Acid:

Addition of 20mM oleic acid to the pre- and final incubation solution inhibited [^{3}H]OA uptake by 95% ($P<0.02$). Uptake of [^{3}H]OA by control muscles (n=7) was 9.5e-7 ± 3.15e-8 mol/mg and by experimental muscles, 5.04e-8 mol/mg (Fig. 5.2; n=2).

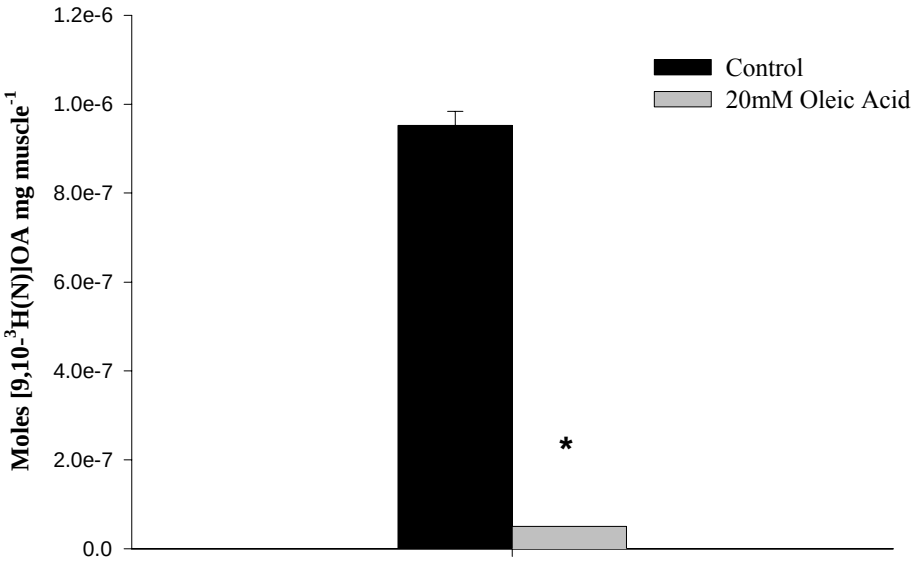


FIGURE 5.2. Competitive Uptake of [^{3}H]OA in the Presence of 20mM Oleic Acid.

Addition of 20mM oleic acid to the pre- and final incubation solution inhibited [^{3}H]OA by 95% (n=2) compared to controls (*n*=7). Data are expressed as the mean of Moles [^{3}H]OA uptake per mg muscle (wet weight). *P<0.02

Inhibition of Oleic Acid Uptake by Glucose:

Radiolabeled oleic acid uptake was significantly ($P<0.02$) inhibited by the presence of 20mM D-glucose in the pre- and final incubation solutions (Fig. 5.3). Control values were 2.2e-6 ± 2.87e-8 mol/mg and experimental values were 1.4e-6 ± 1.73e-7 mol/mg. Inhibition of [^{3}H]OA uptake was 36%.

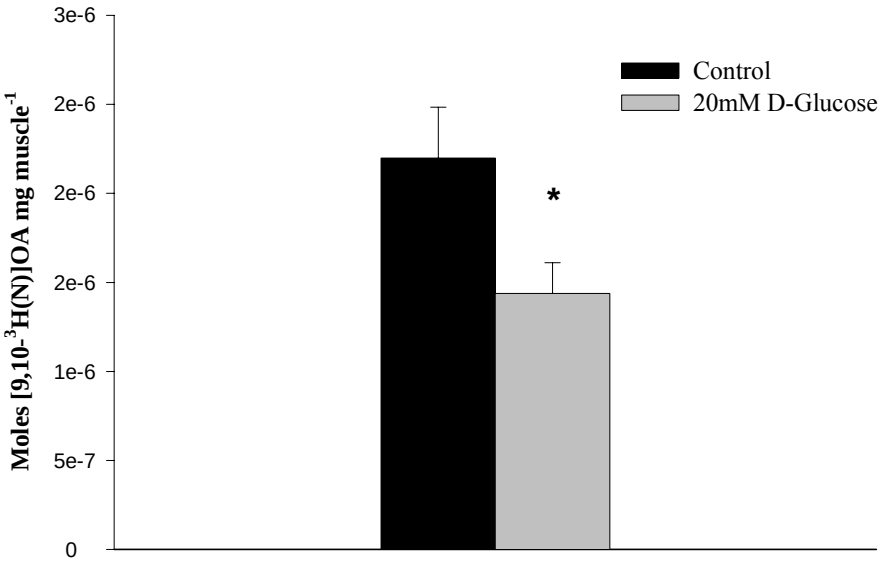


FIGURE 5.3. Inhibition of [^{3}H]OA Uptake by D-Glucose.

Radiolabeled OA acid uptake was significantly inhibited by the presence of 20mM D-glucose in the pre- and final incubation solutions. Inhibition of [^{3}H]OA uptake was 36%. Data are expressed as the mean of Moles [^{3}H]OA uptake per mg muscle (wet weight) ± SEM (*n*=9 paired muscles). *P<0.02

Inhibition of Glucose Uptake by Oleic Acid:

Radiolabeled [^{3}H]2DG transport by the control muscles (0mM oleic acid) was (1.06e-6 ± 3.08e-8 mol/mg). The addition of 1 or 5mM oleic acid had no significant effect on [^{3}H]2DG uptake compared to control muscles (Fig. 5.4). Transport of [^{3}H]2DG was significantly inhibited by 10 and 20mM oleic acid. The addition of 10mM oleic acid to the pre- and final incubations inhibited [^{3}H]2DG transport by 33% (7.1e-7 ± 3.71e-8 mol/mg; $P<0.0005$). The presence of 20mM oleic acid further decreased [^{3}H]2DG transport by 49% (5.4e-7 ± 5.03e-8 mol/mg; $P<0.00005$).

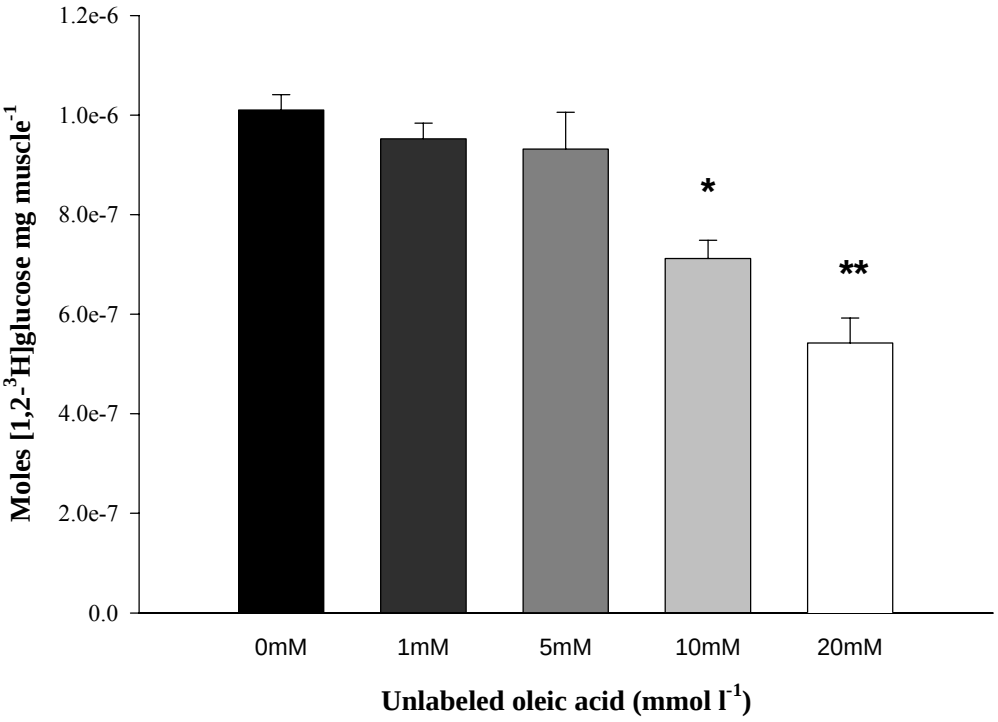


FIGURE 5.4. Inhibition of [^{3}H]2DG Uptake by Oleic Acid.

The addition of 1 or 5mM OA had no significant effect on [^{3}H]2DG uptake compared to control muscles (0mM OA; *n*=33). Transport of [^{3}H]2DG was significantly inhibited by 10 and 20mM oleic acid. The addition of 10mM oleic acid to the pre- and final incubations inhibited [^{3}H]2DG transport by 33%. The presence of 20mM oleic acid further decreased [^{3}H]2DG transport by 49%. Data are expressed as the mean of Moles [^{3}H]2DG uptake per mg muscle (wet weight) ± SEM (*n*=7 paired muscles). *P<0.0005, **P<0.00005

Discussion

Carbohydrate stores in avian muscle and liver tissues power take-off and short flights. Beyond that, most of the energy used by exercising birds is derived from lipid oxidation (Jenni-Eiermann, et al., 2002). Increases in plasma FFA levels correlates directly with increased production of ketone bodies, produced from lipid catabolism (Beylot, 1996). Interestingly, birds have much higher levels of plasma glucose and ketone body concentrations than mammals of comparable body mass (Table 5.1). They also exhibit resistance to insulin-stimulation of glucose uptake by peripheral tissues (Sweazea and Braun, 2005), whereas the uptake of FFA by avian skeletal muscle was enhanced by insulin (unpublished observations). This suggests that birds offer a model of insulin resistance in which to study the effects of carbohydrates and fatty acids on substrate preference as an indicator of potential inducers of insulin resistance.

In the present study, radiolabeled oleic acid ([^{3}H]OA) uptake was decreased 95% ($P<0.02$) in the presence of 20mM OA (Fig. 5.2)and by 36% in the presence of 20mM D-glucose (Fig. 5.3; $P<0.02$). Radiolabeled 2DG uptake was also significantly decreased by 38% in the presence of 20mM glucose (Fig. 5.1; $P<0.001$) and by 49% in the presence of 20mM OA (Fig. 5.4; $P<0.00005$). These data suggest that, at the same concentration, OA is more effective at inhibiting glucose uptake (49%) than glucose inhibition of OA uptake (36%). FFAs provide muscles with more energy per mole (146 ATP/OA) than carbohydrates (36 ATP/glucose). Therefore, it appears that avian muscle utilizes fats as an efficient energy source to support the high metabolic demands of flight.

At the skeletal muscle level, insulin resistance is manifested as an inability of insulin to enhance glucose uptake and glycogen synthesis (Thompson et al., 2000). There are two predominant theories as to the etiology of insulin resistance. The first theory is that high plasma glucose induces it through impaired activation of PKB/Akt, a key step in the insulin signaling pathway (Kurowski et al., 1999). This is a possibility in birds as they display very high plasma glucose levels. The second theory is that high plasma FFA, and their uptake into muscle, induce the observed insulin resistance to glucose uptake. There is convincing evidence that the latter hypothesis is relevant to the situation in birds as they respond to insulin with increased FFA uptake despite being resistant to the glucose lowering effects of insulin and further, they take up significantly more FFA than glucose into skeletal muscle as demonstrated by the results of the present study. In addition, studies have shown that birds preferentially utilize FFA for flight energetics, over glucose (Jenni-Eiermann et al. 2002). It would appear that birds have a protective mechanism against the uptake of excessive glucose as they do not express the insulin-responsive glucose transporter (GLUT4), which in mammals, is responsible for the uptake of large quantities of glucose in response to insulin stimulation (Duclos et al., 1993; Carver et al., 2001; Seki et al., 2003). Moreover, the level of glucose uptake into avian skeletal muscle is minimal and not responsive to common mediators of the insulin and contraction pathways (Sweazea and Braun, 2005).

Further, recent studies have shown that the relationship between intramyocellular lipid concentrations and insulin sensitivity is inverse such that increased uptake of FFA into skeletal muscle decreases the sensitivity of the tissue to insulin-mediated increases in

glucose uptake (Krssak et al., 1999). Therefore, the growing body of research tends to favor the hypothesis that elevated FFA, as opposed to elevated glucose, induce insulin resistance thereby initiating the cascade of pathologies (i.e. hyperglycemia, hyperketonuria and peripheral insulin resistance (IR)) commonly known as type 2 diabetes.

It has been proposed that substrate preference occurs at the level of the sarcolemma (transport) rather than by competition for oxidation (Luiken et al., 2004). Therefore, it would appear that avian skeletal muscle has a greater capacity, and preference, to take up FFA over glucose perhaps due to decreased levels of glucose transport proteins and increased levels of FFA transporters. This is supported by the observation that avian skeletal muscle contains the highest levels of cytosolic fatty acid binding proteins among vertebrates (Guglielmo et al., 1998). It is also possible that the preferential uptake of FFA by avian skeletal muscle induces the notable avian IR to glucose uptake.

Concluding Remarks

The present study supported the hypothesis that elevated FFA inhibit glucose uptake by *in vitro* avian skeletal muscle to a greater extent than the reciprocal inhibition of FFA uptake by glucose.

CHAPTER 6

SUMMARY

The overall goal of the work was to gain a better understanding of how the homeostatic mechanisms of birds have evolved to facilitate the high plasma glucose and free fatty acid levels without the negative consequences that occur in mammals when levels of these substrates are increased above normal for extended periods of time.

Results demonstrate that avian skeletal muscle is resistant to the glucose transport promoting effects of insulin, IGF-1, AICAR (5'aminoimidazole-4-carboxamide-1-βD-ribofuranoside) and caffeine, but responds to phloretin, an inhibitor of protein mediated transport, with decreased uptake suggesting the presence of a glucose transport protein (GLUT). This is supported by maximal glucose uptake occurring at 60 minutes when muscles were incubated over a time course (Ch. 2).

Avian skeletal muscle contains GLUTs 1 and 3, but not GLUT4. Skeletal muscle protein expression of GLUT1 was localized predominantly to areas with abundant vasculature (blood-tissue barriers). Localization of GLUT3 protein could not be determined as an avian antibody does not exist and the mammalian antibodies used did not detect the avian isoform. GLUT1 and 3 gene expression were detected in all tissues examined including skeletal muscle and were found to be highly homologous to the chicken sequences and relatively homologous to human (Ch. 3). The lack of a GLUT4-like insulin responsive transporter in avian skeletal muscle is supportive of the observed inability to enhance glucose uptake using insulin. In addition, the expression of GLUT1

and 3, both basal transporters, supports the findings that glucose uptake in avian skeletal muscle is not inducible by agents that increase GLUT translocation or upregulate transporters already present in the plasma membrane.

Uptake of oleic acid by avian skeletal muscle occurs, at least in part, by a facilitative process as uptake of radiolabeled oleic acid was decreased in the presence of its unlabeled counterpart and by an inhibitor of FAT/CD36 long chain fatty acid transporters (SSO). Skeletal muscle oleic acid uptake was resistant to IGF-1, AICAR and caffeine, but responded to insulin with increased uptake. Interestingly, non-specific inhibitors of protein-mediated transport, phloretin and DIDS, increased oleic acid uptake. These data suggest that other fatty acid transporters (FATP, FABPpm) may be expressed in avian skeletal muscle and/or that a significant amount of diffusion occurs in these tissues (Ch. 4). In reciprocal inhibition studies (Ch. 5), unlabeled glucose and oleic acid inhibited the uptake of their radiolabeled counterparts. Glucose was also shown to decrease labeled OA uptake 36% while OA decreased labeled glucose transport 49%. These studies demonstrated that OA is preferentially taken up by avian muscle.

The studies presented suggest that avian skeletal muscle functions in a way to prevent extensive glucose uptake possibly to prevent oxidative damage. This occurs even in the presence of an array of pharmacological agents that, in mammals, affect the insulin and contraction mediated pathways to increase glucose transport. The uptake of fatty acids, and their subsequent inhibition of glucose uptake, as well as the lack of an insulin responsive glucose transporter, may be involved in the observed resistance to these compounds.

APPENDIX: Animal Subjects Approval

Institutional Animal Care
and Use Committee

THE UNIVERSITY OF
ARIZONA.
TUCSON ARIZONA

P.O. Box 210101
Tucson, AZ 85721-0101

Verification of Review
By The Institutional Animal Care and Use Committee (IACUC)
PHS Assurance No. A-3248-01 -- USDA No. 86-3

The University of Arizona IACUC reviews all sections of proposals relating to animal care and use. The following listed proposal has been granted ***Final Approval*** according to the review policies of the IACUC:

PROTOCOL CONTROL NUMBER/TITLE:

#03-126 - "Substrate Utilization by Avian Skeletal Muscle"

PRINCIPAL INVESTIGATOR/DEPARTMENT:

Eldon J. Braun - Physiology

GRANTING AGENCY:

NSF

SUBMISSION DATE: **August 21, 2003**

APPROVAL DATE: **September 2, 2003** APPROVAL VALID THROUGH*: **September 1, 2006**

*When projects re grant periods extend past the above noted expiration date, the Principal Investigator will submit a new protocol proposal for full review. Following IACUC review, a new Protocol Control Number and Expiration Date will be assigned.

REVIEW STATUS FOR THIS PROJECT WAS CONFIRMED ON: **September 3, 2003**

REVISIONS (if any):

MINORITY OPINIONS (if any):

Richard C. Powell, PhD, MS
Vice President for Research

DATE: September 3, 2003

This approval authorizes only information as submitted on the Animal Protocol Review Form, Amendments, and any supplemental information contained in the file noted as reviewed and approved by the IACUC.

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