GLUCOSE TRANSPORTER-4 IN MONONUCLEAR CELLS OF SLED DOGS

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A

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Abstract

The glucose transporter-4 (GLUT4) is the glucose transporter most responsive to insulin and has been thought to exist predominately in muscle and adipose cells. There have been findings that the glucose transporter-4 isoform is also expressed in subpopulations of white blood cells such as mononuclear cells. This study was designed to validate the presence of GLUT4 in subpopulations of white blood cells of sled dogs and to investigate whether changes in GLUT4 protein levels in white blood cells might be associated with age and stage of conditioning, as it has been reported in muscle. Our initial results have shown the ability to detect GLUT4 in white blood cells of sled dogs with a non-significant trend observed in GLUT4 levels based on age. Subsequent testing showed a statistically significant difference in GLUT4 levels in mononuclear cells based on conditioning in sled dogs. Using sled dogs as a model should enhance our understanding of GLUT4 expression on the surface of subpopulations of white blood cells. The presented projects are groundbreaking for the development of an easy, reliable and minimally invasive diagnostic tool for insulin sensitivity. Our next step in this research is to examine whether the conditioning response of GLUT4 is also observed in human mononuclear cells.

Dedication

"A journey of thousand miles begins with a single step."

Laozi, Chinese philosopher, 604 BC- 531 BC

I have already met so many wonderful people wandering the journey of my life- this thesis is dedicated to those people that decided to accompany my trip for a couple of miles. There have been stages and periods during my odyssey when I needed the encouragement, support and motivation from my closest friends, my parents, my siblings and my mentors to keep taking thousands of single steps. You all have taught me that no single journey is smooth and straightforward and that there are many alternative routes along the way. You have truly inspired me to stay positive, optimistic and open to explore new trails!

Table of Contents

Page

Signature page i

Title page iii

Abstract v

Dedication vi

Table of Contents vii

List of Figures xi

List of Abbreviations xiii

Acknowledgements xv

Chapter 1 1

Introduction 1

1.1 Insulin resistance, obesity, and type 2 diabetes 1

1.2 GLUT4 in mononuclear cells 1

1.3 GLUT4 and exercise 3

1.4 The sled dog model 5

1.5 Summary 5

1.6 References 7

Page

Chapter 2..... 10

Glucose transporter-4 in white blood cells of young and old sled dogs: a model for human biomarker development..... 10

2.1 Abstract 11

2.2 Introduction 12

2.3 Material and Methods 15

2.3.1 Animals and diet 15

2.3.2 Sample collection and preparation 16

2.3.3 Biochemical analysis 16

2.3.4 Statistics 17

2.4 Results 17

2.5 Discussion 18

2.6 Acknowledgements 20

2.7 References 21

Chapter 3..... 25

Conditioning causes increased GLUT4 levels in mononuclear cells of sled dogs..... 25

3.1 Abstract 27

3.2 Introduction 28

3.3 Materials and Methods 31

3.3.1 Animals and diet 31

Page

3.3.2 Blood sampling. 32

3.3.3 Biochemical analysis 32

3.3.4 Statistical analysis of data 33

3.4 Results....34

3.5 Discussion 35

3.6 Acknowledgements 38

3.8 References 39

Chapter 4…… 46

Future Directions……..….. 46

4.1 Refinement of blood sampling protocol 49

4.2 Exercise protocol 51

4.3 Validation in human subjects....52

4.4 Clinical application....52

4.5 References 54

List of Figures

Page

Figure 1.1: Proposed model for the signaling pathway mediating GLUT4 protein expression in skeletal muscle after conditioning…. 3

Figure 2.1: Mean insulin levels of young and old sled dogs…… 24

Figure 2.2: GLUT4 concentrations in WBC of young and old sled dogs…. 25

Figure 3.1**:** GLUT4 concentrations in mononuclear cells of sled dogs is affected by long-term conditioning…… 43

Figure 3.2: Fasting insulin concentrations of conditioned and sedentary dogs indicate an apparent trend of improvement of insulin sensitivity after four months of conditioning… 44

Figure 3.3: Fasting glucose concentrations of conditioned and sedentary dogs indicate an apparent trend of decreased blood glucose after four months of conditioning……..... 45

Figure 3.4: The Homeostasis Model assessment of insulin sensitivity (HOMA-IR) in conditioned dogs is significantly lower than in sedentary dogs.. 46

Figure 3.5: The relationship between GLUT4 levels and the homeostasis model assessment of insulin sensitivity HOMA-IR was investigated using Pearson correlation coefficient…. 47

List of Abbreviations

AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ATF2	Transcription factor 2
ATP	Adenosine triphosphate
ATV	All terrain vehicle
BCA	Bicinchoninic acid
BMI	Body mass index
Ca^{2+}	Calcium ion
CaMKs	Calmodulin-dependent protein kinases
CS	Calf serum
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
GLUT4	Glucose transporter isoform 4
HOMA-IR	Homeostasis Model Assessment of Insulin Resistance
IACUC	Institute of Animal Care and Use Committee
IR	Insulin Resistance
IRS-1	Insulin receptor substrate-1
LKB1	Primary upstream kinase of AMPK
PBS	Phosphate buffered saline
PGC-1α	peroxisome-proliferator-activated receptor-γ coactivator 1α
PI3-kinase	Phosphatidylinositol 3-kinase

POST	Post exercise
p38 MAPK	p38 mitogen activated protein kinase
PRE	Pre exercise
QR code	Quick response code
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT	Room Temperature
T2D	Type 2 diabetes
24H POST	24 hours post exercise
WBC	White Blood Cells

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Chapter 1

Introduction

1.1 Insulin resistance, obesity, and type 2 diabetes

Obesity has increased dramatically in the past decades, more than one-third of U.S. adults are obese (35.7%) [1]. Obesity is one of the greatest risk factors for developing insulin resistance. Insulin Resistance is defined as decreased sensitivity or responsiveness to the metabolic actions of insulin and contributes to the pathophysiology for the development of type 2 diabetes (T2D) [2, 3]. Due to the current epidemiological dimensions of insulin resistance and T2D it is of great interest to carry out research that lends to a better understanding of the molecular mechanisms underlying this major public health problem. Results presented here might help in developing an easy and reliable method for quantifying insulin sensitivity and insulin resistance.

1.2 GLUT4 in mononuclear cells

In 1975, Schwartz et al. were the firsts to report about insulin binding in monocytes, demonstrating that monocytes are the insulin binding cells in subpopulations of mononuclear cells [4]. Mononuclear cells are a subpopulation of white blood cells. Two years later, Beck-Nielsen and Pedersen discovered that the ratio between insulin

binding to monocytes and lymphocytes is constant from person to person, suggesting that it is possible to estimate the insulin binding to monocytes from the binding data obtained from a mixed suspension of mononuclear leucocytes [5]. One year later the same group successfully correlated insulin receptors on monocytes with glucose intolerance and insulin sensitivity [6]. This was the first evidence of biological effects of insulin and insulin binding to monocytes and the group suggested that monocyte receptors for insulin have the same characteristics as insulin receptors in muscle cells and adipocytes [6]. In a different study, the Beck-Nielsen research group confirmed their hypothesis that insulin regulates its own receptor and that insulin binding is affected through intermediate metabolites [7].

The glucose transporter isoform or isoforms expressed by subpopulations of mononuclear cells had not been identified until 2002 when a research group performed a comprehensive analysis of specific high affinity facilitative glucose transporter distribution in human white blood cells and found the glucose transporter (GLUT4) isoform. This group used immunocytochemical detection methods [8]. Next, Maratou et al. showed an increase in translocation of GLUT4 isoforms on the plasma membrane of mononuclear cells after stimulation with insulin and monitored with flow cytometry [9]. While there have been several studies that investigated the presence of GLUT4 expression in mononuclear cells and the effects of insulin using flow cytometry, to our knowledge no research has been performed on exercise induced GLUT4 translocation in mononuclear cells or the quantification of GLUT4 in mononuclear cells using enzyme-linked immunosorbent assay (ELISA) observations. Chapter 2 is focused on the quantification of GLUT4 levels in mononuclear cells of sled dogs using ELISA. In

following up this observation, Chapter 3 stresses the effect of conditioning on GLUT4 protein expression on the surface of mononuclear cells of sled dogs.

1.3 GLUT4 and exercise

It has long been recognized that physical activity and exercise result in many health benefits, especially in disease states, such as insulin resistance and type 2 diabetes (T2D) [10]. Research has shown that conditioning (regular physical activity) increases GLUT4 protein levels in skeletal muscle [11, 12]. It is suggested that this increase of GLUT4 protein levels is insulin independent and contributes to exercise-mediated improvements in insulin sensitivity [10]. There are several adaptations in skeletal muscle due to conditioning and regular exercise: muscle fiber type transformations, increases in mitochondrial activity and content and increases in GLUT4 protein expression [10].

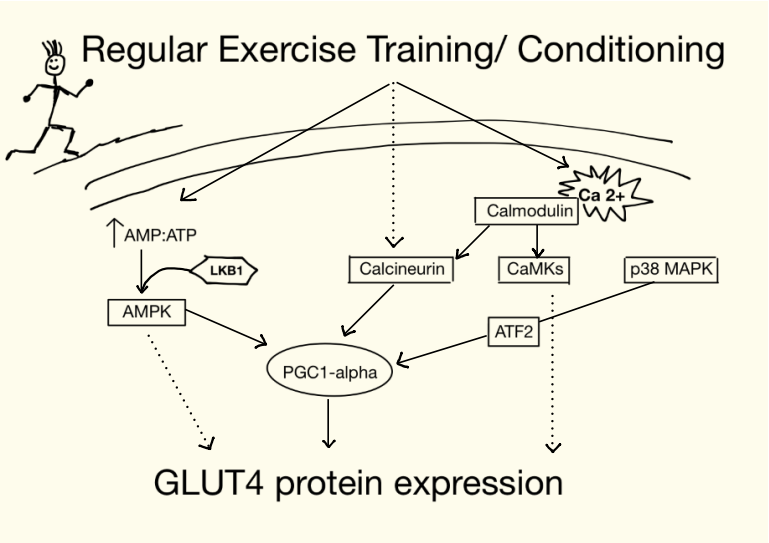

Figure 1.1: Proposed model for the signaling pathway mediating GLUT4 protein expression in skeletal muscle after conditioning. (Adapted from Röckl et al. 2008).

Figure 1.1 proposes a model for the signaling pathways mediating GLUT4 protein expression after endurance training in skeletal muscle. Two pathways appear to act in concert to mediate adaptive responses to conditioning: a. Changes in the cellular energy status (AMP:ATP) and b. in the presence of LKB1 (AMPK kinase), ATP-activated protein kinase is stimulated. It has been suggested by Baar et al. that AMPK may be involved in GLUT4 biogenesis independent and dependent on an increased PGC-1α expression [13]. Regular exercise also leads to an increase in intracellular Ca^{2+} levels, which leads to activation of the Ca^{2+}/calmodulin-dependent phosphatase calcineurin [14] and the Ca^{2+}/calmosulin-dependent protein kinases (CaMKs) [15]. Finally, exercise induced activation of p38 MAPK protein increases PGC-1α expression after activation of ATF2 (transcription factor 2) and might be another key protein in the cellular signaling pathway leading to an increased exercise-induced GLUT4 protein expression [16, 17]. Thus far, all this work on exercise induced GLUT4 translocation has been performed in muscle, which requires invasive muscle tissue biopsies.

In the present study we used mononuclear cells to establish a conditioning response of GLUT-4 on the cell surface as an adaptation to conditioning and regular exercise. A literature review has shown that no study has yet reported changes in mononuclear GLUT4 expression following exercise. Results discussed in Chapter 3 agree with previous reports in skeletal muscle suggesting an increased GLUT4 expression at the plasma membrane of mononuclear cells after long-term conditioning. The biological role of mononuclear cell GLUT4 content remains unclear. More research will be needed to decode the molecular mechanisms leading to an increase in GLUT4 expression in mononuclear cells as result of exercise.

1.4 The sled dog model

Dogs in general are a well-established biomedical research model [18-21]. Sled dogs, in particular, provide a large homogenous sample size, ideal for studying the impact of diet, lifestyle and exercise. The dogs utilized in the following studies were typical racing sled dogs; they were all fed the same diet, housed the same and the conditioned group followed the same exercise regime. All the dogs in this study, both sedentary and conditioned, are genetically similar, selected for performance and are considered elite athletes. The conditioned group of dogs was highly conditioned at the time of the blood draw. They were run on an exercise wheel for 2 hours or hooked in front of an all terrain vehicle (ATV) between 3-5 miles, 5-6 times per week for 4 months prior the study. Some of the dogs from our conditioned group would be part of the mushing team that went on to win the world championship about 5 months later. The sedentary group was also comprised of highly competitive canine athletes, but they did not participate in a regular exercise program for the 4 months prior to sample collection, having completed the competition season in March and not yet commenced preparation for the upcoming racing season.

None of the research presented in chapter 2 and chapter 3, regarding GLUT4 expression in mononuclear cells, has been established in the dog model yet. Our research is an important milestone in making dogs an even more attractive research model. Dogs have been used as a biochemical research model for diabetes for over a century [22]. Although, sled dogs are not a high-risk group for developing T2D, insulin resistance or even obesity, their extreme energy expenditure allows for a unique opportunity for studying glucose metabolism and insulin signaling [23].

1.5 Summary

Chapter 2, *Glucose transporter-4 in White Blood Cells of Young and Old Sled Dogs: a model for human biomarker development*, reports GLUT4 expression in white blood cells of young and old sled dogs. Chapter 3, *Conditioning causes increased GLUT4 levels in mononuclear cells of sled dogs*, reports GLUT4 expression in mononuclear cells of conditioned and sedentary sled dogs. The objective of these projects were to quantify GLUT4 expression on the surface of white blood cells of sled dogs using an ELISA technique and to examine the effects of age and exercise on glucose transport in mononuclear cells. GLUT4 behavior, in regard to insulin stimulation and exercise in skeletal muscle, is a hot topic in diabetes research, yet many questions still exist about insulin resistance. Any efforts that would provide a direct, significantly less invasive, reliable and easy technique for assessing insulin sensitivity and that is also a useful approach for signaling research will likely contribute to the advancement of diabetes research and diagnostics. Using sled dogs as a model should help in this endeavor and enhance our understanding of GLUT4 expression in mononuclear cells as a reliable proxy tissue.

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Chapter 2

Glucose transporter-4 in white blood cells of young and old sled dogs: a model for human biomarker development[1]

2.1 Abstract

The insulin responsive glucose transporter, GLUT4 is found predominantly in muscle and adipose cells. Maratou et al. (2007) reported that there is GLUT4 in white blood cells (WBC) collected from human subjects in response to insulin activation (Maratou et al., 2007). This present study was thus designed to validate the presence of GLUT4 in white blood cells of sled dogs and furthermore to investigate whether changes in levels of the GLUT4 protein might be associated with aging in comparing young versus old sled dogs. Additionally, we examined the blood insulin concentration of both populations of dogs before and after a meal to observe their insulin response. It is well documented in skeletal muscle that GLUT4 expression is increased as a result of conditioning, making sled dogs an excellent model in the Circumpolar North for studying the effects of exercise, nutrition and diabetes (Felsburg, 2002; Kararli, 2006). Blood was withdrawn from 11 healthy sled dogs: 6 young (1-5 years) and physically fit, conditioned for racing and 5 old (7-13 years), retired from racing. The insulin response was determined using blood plasma and ELISA. The buffy coat (containing WBC) was collected with a glass pipette after centrifugation and washed and suspended in 1x

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[1] Polar Record, Schnurr TM, Reynolds AJ, Duffy LK, Dunlap KL. In Press.

phosphate buffer. GLUT4 was measured using ELISA kits (USCN Life Sciences). The results validate that GLUT4 is present in white blood cells in sled dogs. Age had no significant effect in the concentration of GLUT4 between the populations of old and young dogs. A significant difference in insulin levels pre and post meal in young (0.13 $\pm$ 0.03 ng/mL (pre), 0.22 $\pm$ 0.04 ng/mL (post), $p<0.05$) and old (0.13 $\pm$ 0.02 ng/mL (pre), 0.22 $\pm$ 0.03 ng/mL (post), $p<0.05$) dogs was observed, displaying the typical postprandial insulin spike. No significant difference was found in insulin concentration comparing old versus young dogs. Our data shows that sled dogs have quantifiable but non-significant different GLUT4 levels in white blood cells when comparing young (40.4 $\pm$ 2.4 ng/mL) and old (35.3 $\pm$ 8.8 ng/mL) sled dogs ($p>0.05$). Detecting GLUT4 via an ELISA in white blood cells, opens up minimally invasive avenues for studying the underlying molecular mechanisms associated with insulin resistance in more complex, dynamic and physiological systems. This project was the first step in developing this simple, high throughout technique with a potential clinical application for diagnosing insulin resistance.

2.2 Introduction

During the past 20 years, there has been a dramatic increase in obesity in the United States. The incidence of obesity has reached epidemic dimensions, and there are no signs that it will decrease given the current trend. Recent statistics estimate that about one-third of U.S. adults (more than 35%) and approximately 17% (or 12.5 million) of children and adolescents in the age of 2-19 years are obese (Odgen et al., 2012). Obesity is a condition that is characterized by excess body fat and is defined as a body mass

index (BMI) ≥ 30 kg/m^2. The likelihood of becoming obese neither depends on sex, age, or ethnicity, but children who are overweight have an increased likelihood of becoming obese adults (Hedley et al., 2004). The incidence of obesity is undoubtedly an important contributor to the increase in insulin resistance and the metabolic syndrome, as well as in type 2 diabetes (T2D) (Mokdad et al., 2003). Obesity therefore ranks as an independent, high-risk factor for T2D (Storz et al., 1999).

The principal role of the hormone insulin is to mediate the redistribution of the GLUT-4 isoform from an intracellular vesicle pool into plasma membranes of insulin-responsive tissues and thus regulating the uptake of glucose. In the presence of insulin stimulation, the translocation of GLUT4 to the membrane surface is necessary for glucose transport into the cell (Abel et al., 2003). Elevated plasma glucose concentrations stimulate pancreatic ß-cell secretion of insulin (Viscarra et al., 2011). Insulin binds to the insulin receptor (IR) on target tissues and stimulate the phosphorylation of insulin receptor substrate-1 (IRS-1). IRS-1 associates with phosphatidylinositol 3-kinase (PI3-kinase). After association of these two substrates, the phosphorylation of Akt2 takes place (Czech, 1995; Heller-Harrison et al., 1995; Leney and Tavaré, 2009) causing the translocation of GLUT4 to the plasma membrane, increasing the uptake of glucose and decreasing plasma glucose levels. (Czech, 1995; Leney and Tavaré, 2009).

GLUT4 has a central role in whole-body glucose homeostasis and defective GLUT4 trafficking might represent one of the earliest defects contributing to insulin resistance in humans (Stöckli et al., 2011). Insulin resistance is characterized by an inability of cells to respond to insulin upon stimulation with glucose and presents as an important risk factor for the development of T2D (Bastard et al., 2006). GLUT4 is the

glucose transporter most responsive to insulin and is found predominantly in muscle and adipose cells. Quantification of GLUT4 would then require invasive biopsies. A transformative study done by Maratou et al. (Maratou et al., 2007) demonstrated that there is GLUT4 in insulin stimulated white blood cells (WBC) collected from human subjects in response to insulin activation. In a subsequent study this research group further validated the use of insulin-stimulated GLUT4 expression in mononuclear cells as a reliable diagnostic tool by correlating these levels with HOMA-IR in diabetic patients (Maratou et al., 2009). Hence, this technique would seem to offer great potential as a relatively non-invasive diagnostic tool and creates unique possibilities for studying the molecular basis for T2D (Zorzano et al., 2005).

Historically, dogs have played a critical role in our understanding and treatment of diabetes and scientists have used dogs as a biochemical research model for diabetes for over a century (Catchpole et al., 2005). Though there are species-specific pathologies associated with diabetes, dogs develop insulin dependent and independent forms of diabetes, and gestational diabetes akin to humans (Bergman et al., 2006; Catchpole et al., 2005; Johnson, 2008). The prevalence of canine diabetes (classified into insulin deficiency diabetes) is significantly lower than human, which could be a result of better diagnostics or an increased incidence of risk factors like obesity, as seen in humans, or both, but an increasing trend has been observed (Catchpole et al., 2005). Dogs are a proven model for biochemical research (Dunlap et al., 2006; Greeley et al., 2001; Milgram et al., 2002), and can be an innovative model to link activity and nutrition to the physiological and immune effects seen in metabolic syndrome and related disorders. For the Circumpolar North, racing sled dogs are excellent models for studying

health effects related to exercise, nutrition and metabolic syndrome (Felsburg, 2002; Kararli, 2006). Nutritional intervention and exercise has shown to improve insulin sensitivity and increase GLUT4 expression (Carey and Kingwell, 2009; Ruel and Couillard, 2007). The main purpose of this pilot study was to validate quantifiable amounts of GLUT4 in white blood cells of dogs using a simple commercially available ELISA and furthermore compare GLUT4 levels in young versus old sled dogs. Additionally, we examined the blood insulin response to a meal.

2.3 Material and Methods

2.3.1 Animals and diet

Sled dogs, raised in Salcha, Alaska (Latitude 65°N, 147°W) were used as test subjects. All procedures were reviewed and approved by the Institute of Animal Use and Care Committee at the University of Alaska Fairbanks (IACUC, #02-14). The dogs that were used were typical racing sled dogs that were evenly distributed for sex. Both populations of dogs, healthy young racing sled dogs (n=6) and healthy old retired sled dogs (n=5), were from the PileDriver Kennel in Salcha, AK. The age distribution for YOUNG dogs ranged from 1 to 5 years (3.2 years $\pm$ 1.8) and for OLD dogs ranged from 7 to 13 years (10 years $\pm$ 2.1). All dogs were sexually intact. Housing arrangements consisted of 2-m chains on which the dogs were tethered for the duration of the study. Each dog had access to his or her own house. Dogs in both groups were fed the same diet (Purina Pro Plan Performance) and were allowed ad libidium access to water. Each dog was fed to maintain its ideal body condition score of 3 (Laflamme, 1997).

2.3.2 Sample collection and preparation

All dogs were sampled pre meal (PRE) to measure fasting plasma insulin levels and GLUT4 and post meal (POST) for plasma insulin levels. Blood samples (4mL) were collected via cephalic venipuncture into prechilled EDTA-treated vacutainer sample tubes containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Tubes were stored upright at room temperature for no longer than 2 hours prior to centrifugation and centrifuged for 15 min at 3600 RPM at room temperature. For the insulin analysis, the plasma was transferred into freezer vials, flash frozen in liquid nitrogen and immediately stored at -80˚C. For the GLUT4 analysis, the buffy coat (mononuclear Interphase layer containing white blood cells) was collected, washed in 1x phosphate buffer saline twice, stimulated with 100nM insulin for 20 minutes, sonicated, transferred into freezer vials, flash frozen in liquid nitrogen and stored at -80˚C.

2.3.3 Biochemical analysis

The concentrations of GLUT4 in white blood cells, were measured with a commercially available ELISA (USCN Life Science Inc., United States). The USCN Life Science GLUT4- ELISA kit is a sandwich enzyme immunoassay for the in vitro quantitative measurement of GLUT4 in canine tissue homogenates and other biological fluids. The micro filter plate in the kit has been pre-coated with a monoclonal antibody specific to GLUT4. GLUT4 concentrations of the samples were then determined by further extrapolation from a standard curve developed from known concentrations of GLUT4. After appropriate sample and standard dilution, the procedure supplied with the assay was followed. GLUT4 concentrations were determined by comparing the optical

density (read spectrophotometric with a microplate reader at 450nm) of each sample to the standard curve. The plasma concentrations of insulin (Porcine/Canine; ALPCO, Salem NH) were measured with a commercially available ELISA. The ALPCO Insulin ELISA is also a sandwich type immunoassay. Monoclonal antibodies specific for insulin are immobilized to the 96-well microplate as the solid phase. Sample concentration was determined by extrapolation from a standard curve. Again, the procedure supplied with the assay was followed. Optical Density was measured with a microplate reader at 450nm and reference wavelength at 620nm.

2.3.4 Statistics

The curves were constructed using Prism5 Software (GraphPad Software, La Jolla, CA). All samples were analyzed in duplicates and run in a single ELISA kit. PRE and POST meal insulin means $\pm$ SE were compared between Young and Old Sled dogs using a paired t-test and considered significantly different at $P<0.05$. GLUT4 means $\pm$ SE were compared between Young and Old dogs using an unpaired student's t-test and considered significantly different at $P<0.05$. Outliers were determined using Grubbs' test (significance levels $\alpha=0.05$).

2.4 Results

Insulin Concentrations increased post meal. There was a 59% increase in mean plasma insulin pre and post meal for both dog populations, regardless of age. Pre and post meal insulin was increased significantly in young and old dogs (Fig. 2.1). Age had no effect on Insulin levels.

GLUT4 levels in white blood cells. GLUT4 was quantified in white blood cells of sled dogs. The GLUT4 concentration of young dogs was 40.4 (± 2.4 ng/mL). GLUT4 concentration of old dogs was 35.3 (± 8.8 ng/mL). Age had no significant effect in the concentration of GLUT4 between the populations of old and young dogs (Fig 2.2). There was one outlier in each population of dogs so the number was reduced to n=5 (young dogs) and n=4 (old dogs).

2.5 Discussion

Currently, the cellular mechanisms underlying insulin resistance and T2D are often studied in vitro using myocytes and adipocytes, or in vivo with invasive muscle or adipose biopsies. Our data support the results of Maratou (Maratou et al., 2007) and indicates that there is significant and quantifiable activity of GLUT4 in WBC of sled dogs in response to insulin, a finding that opens up many opportunities for understanding the molecular mechanisms associated with insulin resistance and of particular interest, a minimally invasive diagnostic tool. A major hindrance to proper diagnosis and treatment of insulin resistance lies in the fact that there is currently no direct method for determining insulin resistance that has a widespread clinical application. Insulin resistance is typically diagnosed with a combination of comorbidities and mathematical formulation based on glucose-insulin ratios, such as HOMA-IR (Ascaso et al., 2003; Olatunbosun and Dagogo-Jack, 2011). This approach severely compromises the reliability of diagnosis, especially during the early onset of insulin resistance, when treatment and lifestyle changes would likely be most effective.

This study focused on sled dogs, incredible athletes, which provide a homogenous population for studying environmental impacts such as nutrition and exercise on blood parameters (Dunlap et al., 2006; Olatunbosun et al., 1997; Reynolds et al., 1999). Even the older sled dogs in this study are relatively fit compared with other canine models in our study group (Dunlap et al., 2006). While older dogs are no longer competitive, they still remain physically fit because they are routinely used to teach younger animals. Because of the uniformity and rigorous exercise regime, sled dogs provide a unique model for studying insulin signaling in response to exercise and maybe even age, but may not be the perfect model for obesity and diabetes. The results of this study indicate that sled dogs exhibit a typical insulin spike after a meal, which further indicates that sled dogs provide a reliable model for normal insulin response.

Perhaps the most important finding of this study was a non-significant, but apparent trend in GLUT4 in WBC with aging. This has also been observed in other species in muscle (Kern et al., 1992). This trend may become significant if expression on the cell surface, rather than overall concentration is considered. The main objective of this project was to see whether significant and quantifiable amounts of GLUT4 could be detected in WBC of dogs. Now that we have established this, the next step is to assess expression of GLUT4 and correlate GLUT4 levels with HOMA-IR. We are now developing and refining this technique to allow for a fast, reliable, and simple method for quantifying GLUT4 expression on the cell surface. While our findings are quite preliminary, our results are very promising. Sled dogs are proving to be a fantastic model for insulin signaling because exercise and conditioning has a well-established effect on GLUT4 levels (Ebeling et al., 1993). Furthermore, the energy needs and

expenditure of a sled dog is 3- 8 times that of the most elite human athlete (Hinchcliff et al., 1997). Our future studies will benefit from comparisons with the established conditioning response in muscle to further validate this technique.

2.6 Acknowledgements

We are grateful for the assistance and support of PileDriver Kennels and Jing Zheng for helping with the sample collection. This project was partly funded by the Department of Chemistry & Biochemistry, UAF and Nestle-Purina, St. Louis, MO. This publication was made possible by grants from the National Center for the Research Resources (5P20RR016466) and the National National Institute of General Medical Sciences (8P20GM103395-12), from the National Institutes of Health. It's contents are the sole responsibility of the authors and do not necessarily represent the official view of NCRR or NIH.

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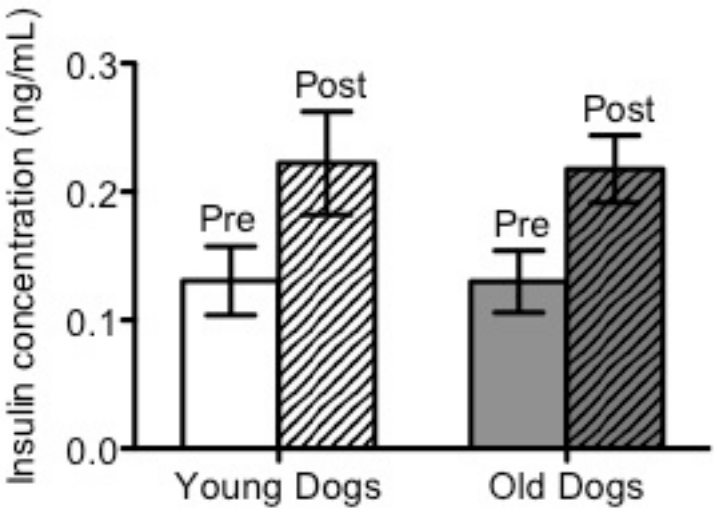

Figure 2.1: Mean insulin levels of young and old sled dogs. Insulin levels for young dogs were: pre meal 0.13 (± 0.03 ng/mL, n=6); post meal 0.22 (± 0.04 ng/mL, n=6); $p<0.05$. Insulin Levels for old dogs were: pre meal 0.13 (± 0.02 ng/mL, n=5); post meal 0.22 (± 0.03 ng/mL, n=5); $p<0.05$. Insulin level means pre and post meal are significantly different at the 95% confident level in old and in young dogs ($p<0.05$). There was no age-induced difference in insulin levels in sled dogs ($p>0.05$).

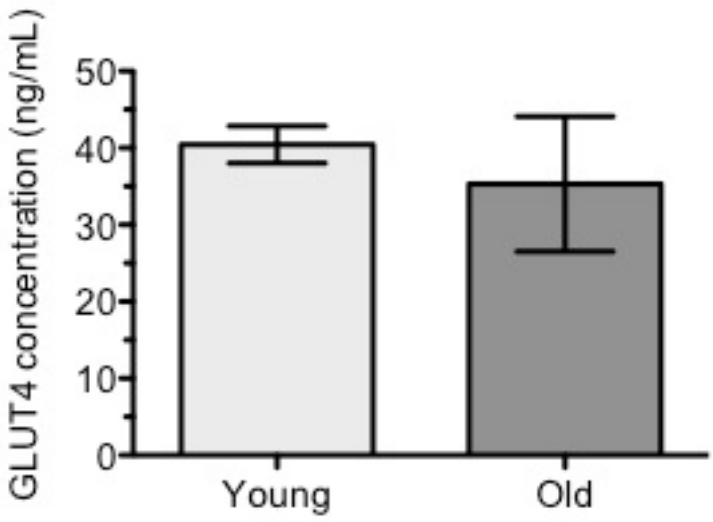

Figure 2.2: GLUT4 concentrations in WBC of young and old sled dogs. GLUT4 in WBC of Young Dogs was 40.4 (+ 2.4 ng/mL, n=5) and Old Dogs 35.3 (+ 8.8 ng/mL, n=4). Means are not significantly different at the 95% confidence level between Young and Old Dogs ($p > 0.05$).

Chapter 3

Conditioning causes increased GLUT4 levels in mononuclear cells of sled dogs[1]

3.1 Abstract

Exercise is a corner stone therapy for mitigating type 2 diabetes (T2D) and insulin resistance. This study was designed to investigate the effects of physical conditioning on the expression of the insulin sensitive glucose transporter, GLUT4 protein on mononuclear cells in dogs as compared to results reported in human skeletal muscle and the skeletal muscle of rodent models. Additionally, we examined the plasma insulin and blood glucose concentrations for the Homeostatic Model Assessment (HOMA-IR). GLUT4 expression in mononuclear cells was measured using a commercially available enzyme-linked immunosorbent assay (ELISA). Our results indicate that the state of conditioning had a significant effect on the GLUT4 expression at the surface of mononuclear cells, with the conditioned dogs (6690 ± 184 ng/g protein) having significantly higher GLUT4 levels as compared to the sedentary group (4290 ± 773 ng/g protein, $p<0.05$). HOMA-IR was also affected by conditioning in dogs ($p<0.05$). GLUT4 levels in mononuclear cells of sled dogs were inversely correlated with the homeostasis model assessment of insulin sensitivity (HOMA-IR) ($r=-0.70$, $p<0.05$). This study demonstrates that conditioning increases GLUT4 levels in mononuclear cells of

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[1] Journal of Applied Physiology, Schnurr TM, Reynolds AJ, Gustafson SJ, Duffy LK, Dunlap KL. Submitted.

sled dogs as it has been previously reported in skeletal muscle. These results show the same in GLUT4 levels in mononuclear cells that is reported in skeletal muscle. Our results support the potential of white blood cells as a proxy tissue for studying insulin signaling and may lead to development of a minimally invasive and direct marker of insulin resistance. This may be the first report of GLUT4 in mononuclear cells in response to exercise and measured with ELISA.

3.2 Introduction

GLUT4 is found predominately in muscle and adipose tissue [1]. GLUT4 has a central role in whole-body glucose homeostasis and defective GLUT4 trafficking likely represents one of the earliest defects contributing to insulin resistance in humans [2]. Insulin resistance is characterized by an inability of cells to respond to insulin upon stimulation with glucose and presents as an important risk factor for the development of T2D [3]. Studies have linked insulin resistance as a strong predictor of T2D [4].

Exercise is a well-documented modulator of GLUT4 and is used as a tool to study GLUT4 trafficking. Regular exercise has health promoting effects and much research is directed at better understand the molecular mechanisms underlying these beneficial effects to identify new therapeutic targets [5]. Exercise training has been shown to mediate skeletal muscle enzymes, transcription factors, transporters and chaperones through an adaptive response to chronic training [5]. The exercise-induced response to insulin occurs by recruiting more GLUT4 to the cell surface from a larger total muscle pool of GLUT4 [6]. The resulting increase in muscle GLUT4 protein is associated with an increased capacity for glucose transport [7, 8]. Similar findings have

been reported in rats [9]. Several studies have reported that the adaptive increase in the GLUT4 protein in muscle cells occurs as early as the first week of exercise training [10, 11]. While there are findings that show an adaptive increase in the GLUT4 protein expressed in muscle cells, it remains unclear if exercise also increases GLUT4 expression in other tissues such as in white blood cells (WBC), the focus of this current study.

Insulin resistance is typically diagnosed with a combination of comorbidities and a mathematical index based on fasting glucose-insulin ratios, such as the homeostatic model assessment called HOMA-IR [12]. The HOMA-IR model has become the most widely used clinical and epidemiological tool for assessing insulin sensitivity, since it requires only a single plasma sample assayed for insulin and glucose [12]. Hence it is considered an indirect measure of insulin sensitivity employing an approximation described by a simple equation as index of insulin resistance [13]. Any strategy that quantifies GLUT4 would thus be a powerful direct measurement and diagnostic tool for early on-set of insulin resistance. This technique is currently limited in its application since it requires invasive biopsies of muscle and/or fat tissue and is therefore unsuitable for clinical application and epidemiological studies. A transformative study by Maratou et al. demonstrated that an increase in GLUT4 in WBC collected from human subjects in response to insulin activation, measured with a flow cytometer [14]. In a subsequent study this research group reported a negative correlation between GLUT4 expression in mononuclear cells and HOMA-IR in diabetic patients using flow cytometry [15]. Hence, WBC as a proxy would seem to offer great potential as a minimally invasive diagnostic tool requiring only a single blood sample and might be a more sensitive indicator than

current clinical applications, creating unique possibilities for studying the molecular basis for T2D.

Historically, dogs have played a critical role in our understanding and treatment of diabetes, and scientists have used dogs as a biochemical research model for diabetes for over a century [16]. Though there are species-specific pathologies associated with diabetes, dogs develop insulin dependent and independent forms of diabetes, and gestational diabetes akin to humans [16-18]. The prevalence of canine diabetes (classified into insulin deficiency diabetes) is significantly lower than humans, which could be a result of better diagnostics in humans or an increased incidence of risk factors like obesity, or both, but an increasing trend in dog has also been observed [16]. Dogs are a proven model for biochemical research [19-21] and could prove to be an equally effective model linking activity and nutrition to the physiological and immune outcomes seen in metabolic syndrome and related disorders.

While our preliminary experiments were aimed at determining whether GLUT4 levels could be detectable in WBC of sled dogs using a commercially available ELISA (chapter 2), the main purpose of this present study was to evaluate this technique further by detecting the expression of GLUT4 on mononuclear cells in response to conditioning, as it is well reported in skeletal muscle. The second goal of this study was to assess the utility of assaying GLUT4 levels in mononuclear cells as a measure of insulin sensitivity by correlating GLUT4 levels with HOMA-IR. We hypothesized that GLUT4 levels in mononuclear cells will increase in response to endurance exercise training and be negatively correlated with HOMA-IR and other comorbidities of insulin resistance, therefore presenting a more sensitive index of insulin resistance. If such a trend exist in

this homogenous population, the next step will be to measure conditioning response in human subjects.

3.3 Materials and Methods

3.3.1 Animals and diet

Sled dogs, raised in Salcha, Alaska (Latitude 65°N, 147°W) were used as test subjects. The Institute of Animal Care and Use Committee (IACUC) at the University of Alaska Fairbanks approved the protocol of this study (#02-14). The dogs used were typical racing sled dogs from similar lineage; eight conditioned dogs (n=8) and eight sedentary dogs (n=8) were sampled. The conditioned dogs were exercised 4 to 6 days per week for a minimum of four months prior the experiment and were following a uniform training protocol. The sedentary dogs were not conditioned for the four months prior to the experiment but were at the onset of their training season when sampling occurred. Groups were balanced for ability and age [conditioned, range 1.5 to 6 years (3 ± 2 years); sedentary, range 1.5 to 6 years (3 ± 2 years)]. The sedentary group had slightly more males (n=6), while the conditioned group had slightly more females (n=6). All dogs were sexually intact. Housing arrangements consisted of 2-m chains on which the dogs were tethered for the duration of the study. Each dog had access to his or her own house. Dogs in both groups were fed the same diet (Purina Pro Plan Performance) and were allowed ad libitum access to water. Each dog was maintained at an ideal body condition score of 3 [22]. The temperature range on the day of the experiment was 4-7°C (September 5th, 2012).

3.3.2 Blood sampling.

All samples were collected after an overnight fast between 9 and 10 am. Blood was collected into EDTA tubes (6mL, for measurement of insulin and glucose concentrations) and BD Separation tubes (6mL, for measurement of GLUT4 levels) using the cephalic vein. All tubes were stored upright at room temperature until centrifugation. Whole blood was spun within 2 hours of blood collection at room temperature at 3600 rpm for 15 min. Aliquots of plasma collected in EDTA tubes were immediately frozen at –80°C for later insulin and glucose analysis. The buffy coat (mononuclear interphase layer containing white blood cells) was collected using BD mononuclear separation tubes, resuspended in 3mL of RPMI w/ 5% calf serum and centrifuged for 15 min at 1500 RCF. Mononuclear cells were washed a total of three times and resuspended in 4mL of RPMI w/ 5% calf serum. Aliquots of the resuspended sample were used for GLUT4 ELISA analysis and adjusted for protein content using BCA Protein assay.

3.3.3 Biochemical analysis

GLUT4 levels at the surface of mononuclear cells were assessed using a commercially available ELISA (USCN Life Science Inc., United States) according to manufacturer's instruction and absorbance was read at 450 nm. To ensure GLUT4 levels were indicative of surface amounts, levels were compared with samples that were sonicated. Sonicated samples had 3 fold higher GLUT4 levels compared with unsonicated samples (chapter 2). A BCA Protein Assay (Pierce, Theroms Scientific,

United States) was used for protein adjustment in regard with GLUT4 and absorbance was read at 562 nm. Insulin levels were measured using ELISA (Porcine/Canine; ALPCO, Salem NH), following the protocol by the manufacturer and taking absorbance readings at 450nm. All absorbance readings were done using Synergy HT multi-mode microplate reader (BioTek, United States). Plasma Glucose analysis was performed by the North Pole Veterinary Clinic (North Pole, AK) using the in-house diagnostic Catalyst® Chemistry Analyzer (IDEXX, United States). HOMA-IR was calculated for each individual with the linear approximation formula, which divides the product of insulin (in μU/mL) and glucose (in mmol/L, data not shown) concentrations by 22.5 [23]. All samples were assayed in duplicates as an internal control.

3.3.4 Statistical analysis of data

Samples were analyzed using GraphPad Prism statistical software (version 5.0) to evaluate significance between conditioned and sedentary populations. Outliers were determined using Grubbs' test (significance level alpha=0.05). There were two dogs (one in the conditioned group, one in the sedentary group) that had plasma insulin levels that were significant outliers when compared with the other dogs within their population, respectively. We removed the data for both dogs for analysis involving fasting insulin levels and calculation of HOMA-IR. Additionally, there was a dog in the conditioned group that we experienced difficulties in collecting the buffy coat. As expected, this dog showed GLUT4 levels that were significantly lower than the rest of the population even after adjusting for the amount of protein in the sample. We removed data for this specific dog for further data analysis regarding GLUT4 levels. The differences between physiological variables before and after endurance training were evaluated by an

unpaired Student's t-test. Pearson's Product-Moment correlation coefficient (r) was used to examine the linear correlation between the two variables: HOMA-IR and GLUT4. Differences were considered significant at $P \leq 0.05$. All data are reported as means ± SD.

3.4 Results

GLUT4 levels in Mononuclear Cells: Dogs that were conditioned for four months prior to the experiment had significantly higher GLUT4 levels as compared to the population of dogs that did not receive conditioning (Fig. 3.1, $p<0.05$). GLUT4 concentrations in mononuclear cells of conditioned dogs were 56% higher than sedentary dogs (6690 (± 184 ng/g protein) vs. 4290 (± 773 ng/g protein)). In addition, conditioned dogs not only had significantly higher GLUT4 levels at rest but also display less variability as compared to the sedentary dogs (Fig. 3.1).

Plasma Insulin concentrations: Sedentary dogs showed a trend towards higher insulin levels, 3.8 (± 0.69 μU/mL) as compared to the conditioned group, 3.3 (± 0.23 μU/mL) but means were not statistically different (Fig. 3.2, $p>0.05$).

Plasma Glucose concentrations: Sedentary dogs showed a trend towards higher glucose levels, 110 (± 3 mg/mL) as compared to the conditioned group, 96 (± 5 mg/mL). Means were statistically different (Fig. 3.3, $p<0.05$).

Homeostasis Model assessment of insulin sensitivity (HOMA-IR): The sedentary dogs had 35% higher HOMA-IR as compared to the conditioned dogs (0.76 (± 0.10) vs. 1.03 (± 0.24); $p<0.05$, Fig. 3.4). HOMA-IR was calculated for each individual dog using fasting insulin and glucose levels in a resting state, prior to any exercise. HOMA-IR is

calculated by dividing the product of insulin (in μU/mL) and glucose (in mmol/L) concentrations by 22.5.

Correlation of HOMA-IR and GLUT4 levels in mononuclear cells: HOMA-IR calculated with the linear formula was negatively correlated with GLUT4 levels in all dogs across the study population (n=13, r= -0.70, P=0.01, Fig. 3.5). Sedentary dogs showed higher HOMA-IR and lower GLUT4 levels on the plasma membrane of mononuclear cells compared to conditioned dogs.

3.5 Discussion

To our knowledge this is the first report of GLUT4 in mononuclear cells of dogs in response to conditioning and the first time GLUT4 expression on the surface of mononuclear cells has been reportedly measured with an ELISA, in any species. We have demonstrated that the state of conditioning had a significant effect on the concentration of GLUT4 levels in mononuclear cells (Fig. 3.1). This agrees with previous reports in muscle, demonstrating that an increase in GLUT4 protein expression is an important chronic adaptation to exercise training, parallel to an increase in mitochondria and oxidative capacities and the transformation of muscle fiber types [24].

Our results suggest that four months of exercise training lead to improved insulin sensitivity as indicated by a lower HOMA-IR (Fig. 3.4). This was further substantiated with a trend towards reduced insulin levels (Fig. 3.2) and significantly lower glucose levels (Fig. 3.3), despite a corresponding increase in GLUT4 levels. The overall higher GLUT4 expression in mononuclear cells in conditioned dogs is likely a result of chronic adaptation to conditioning, a conclusion that is well supported by studies in human

skeletal muscle [25]. There is recent evidence from rodent models suggesting that the training-related increase in glucose transport and GLUT4 expression is a function of an increase in the number of glucose transporters within the muscle plasma membrane and that enhanced insulin-stimulated glucose transport does not involve a change in the individual glucose transporter activity [26].

Previous studies of GLUT4 in mononuclear cells reported GLUT4 values in arbitrary units using flow cytometry after stimulating the cells with insulin [14]. This technique is the gold standard for measuring GLUT4 in muscle but has further limited application for diagnostics because of the elaborate equipment required and the need for same day analysis. We reported baseline GLUT4 expression on mononuclear cells without insulin stimulation using a commercially available ELISA. This application has an exciting potential as a diagnostic tool and may further our understanding of the molecular mechanism(s) underlying insulin resistance.

While sled dogs may not be the ideal model for obesity and T2D, they do appear to be an ideal model for insulin signaling in regard to exercise. Exercise and conditioning has a well-established effect on insulin sensitivity and GLUT4 expression [27]. The absolute energy needs and expenditure of a racing sled dog during exertion in the cold is 3- 8 times that of most elite human athletes [28]. Sled dogs are incredible athletes that provide a homogenous population for studying environmental impacts such as nutrition and exercise on blood parameters [21, 29, 30]. It should be emphasized that even the sedentary sled dogs in this study are relatively fit compared with other canine models [21]. While the sedentary dogs did not follow a rigorous conditioning protocol during the four months prior to the study, it is important to reiterate that they were at their

on-set of training for the upcoming mushing season and had competed competitively in the previous racing season. The sedentary dogs started a training program the day after the blood draw to prepare for the upcoming season. Observing differences in GLUT4 in such a homogenous population without insulin stimulation is support that differences will likely be more dramatic in disparate population: Values for instance between people with insulin resistance will likely have significantly reduced GLUT4 from unaffected populations. Another marker for insulin resistance and T2D that is becoming increasingly popular in the clinical setting is glycosylated hemoglobin (HbA1c) [31]. While we observed significant differences in HOMA-IR, no changes were observed in HbA1c between groups (data not shown). HOMA-IR was originally developed and validated for the use in humans and includes assumptions that might not hold true for all animals [12], but the assumptions used in HOMA-IR analysis have shown to largely hold true in dogs [32].

A simple GLUT4 ELISA that reports concentration would be helpful in nutritional intervention studies and in monitoring exercised induced muscle damage during T2D exercise therapies [9, 29, 33]. Our results support the extensive body of research in muscle tissues and substantiates evidence that mononuclear cells are an excellent proxy for assessing insulin sensitivity with a simple low-invasive technique. Our future studies will benefit from further comparisons with the established conditioning response in muscle to further validate our technique and the use of mononuclear cells. While, the obvious and eventual goal is to use this approach in a human clinical study, the use of a sled dog model has not only offered a platform to refine our technique but has validated our direction by detecting changes in an extremely homogenous population.

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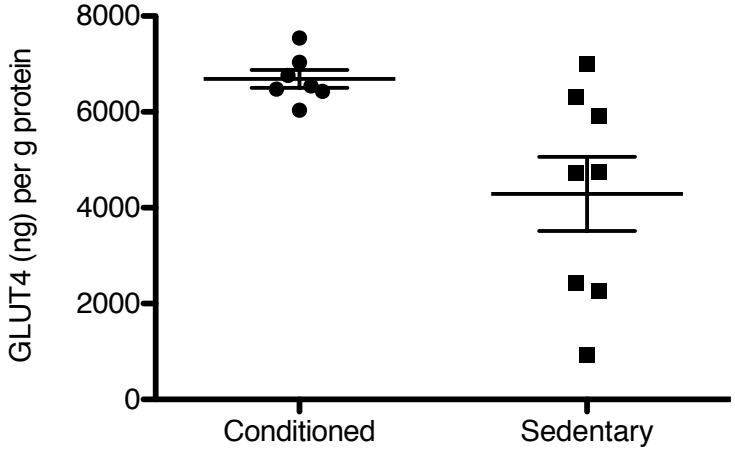

Figure 3.1**:** GLUT4 concentrations in mononuclear cells of sled dogs is affected by long-term conditioning. GLUT4 concentrations were measured via ELISA in mononuclear cells of conditioned (n=7) and sedentary (n=8) sled dogs. GLUT4 in mononuclear cells of conditioned dogs was 6690 (± 184 ng/g protein) and in sedentary dogs was 4290 (± 773 ng/g protein). Means were statistically different (t-test, $p<0.05$).

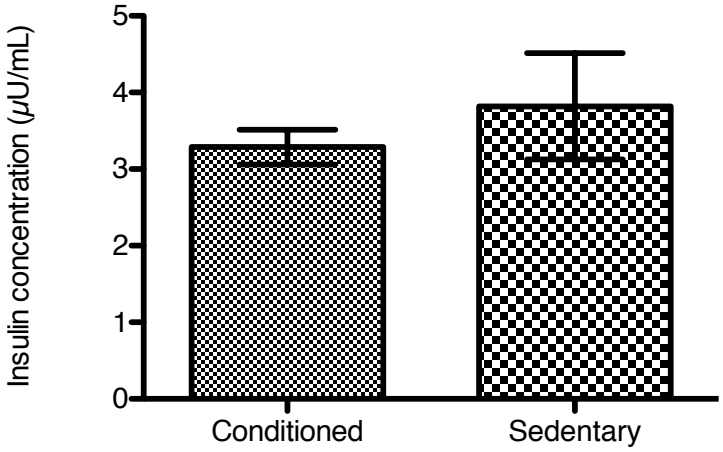

Figure 3.2: Fasting insulin concentrations of conditioned and sedentary dogs indicate an apparent trend of improvement of insulin sensitivity after four months of conditioning. Insulin concentrations were measured via ELISA using the blood plasma in conditioned (n=7) and sedentary (n=7) sled dogs after an overnight fast. Insulin concentration of conditioned dogs was 3.3 (± 0.23 μU/mL) and in sedentary dogs 3.8 (± 0.69 μU/mL). Although data show an apparent trend, means were not significantly different (t-test, $p>0.05$).

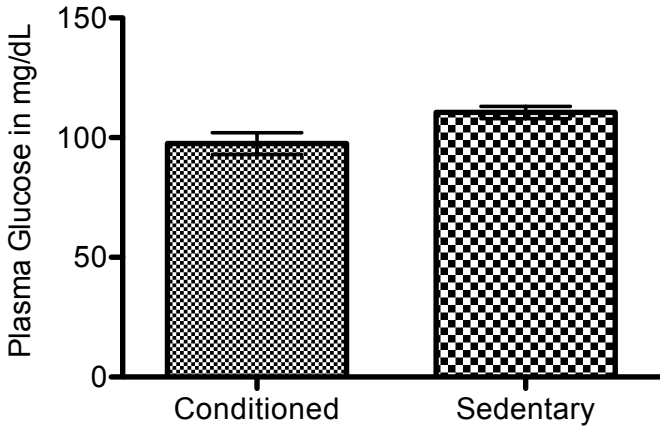

Figure 3.3: Fasting glucose concentrations of conditioned and sedentary dogs indicate an apparent trend of decreased blood glucose after four months of conditioning. Glucose levels were measured using the blood plasma in conditioned (n=7) and sedentary (n=7) sled dogs after an overnight fast. Glucose concentration of conditioned dogs was 96 (± 5 mg/dL) and in sedentary dogs 110 (± 3 mg/dL). Means were significantly different (t-test, $p<0.05$).

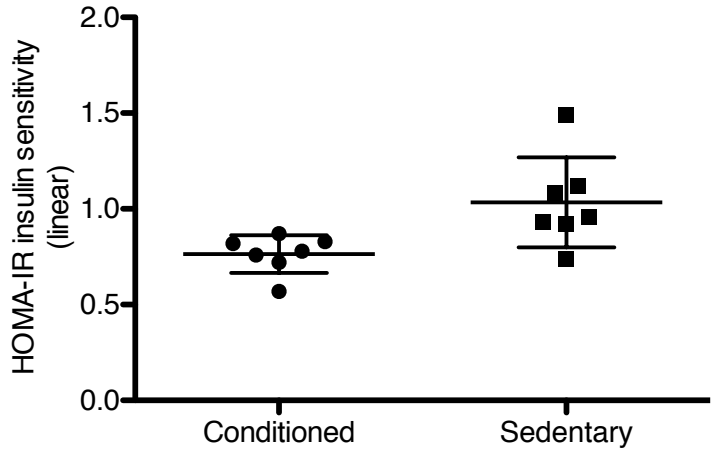

Figure 3.4: The Homeostasis Model assessment of insulin sensitivity (HOMA-IR) in conditioned dogs is significantly lower than in sedentary dogs. HOMA-IR was estimated with the linear model using fasting insulin and glucose levels. HOMA-IR was affected by conditioning in dogs. The four months conditioned dogs had significantly lower HOMA-IR (0.76 ± 0.10) as compared to the sedentary dogs (1.03 ± 0.24). Means were statistically different (t-test, $p<0.05$).

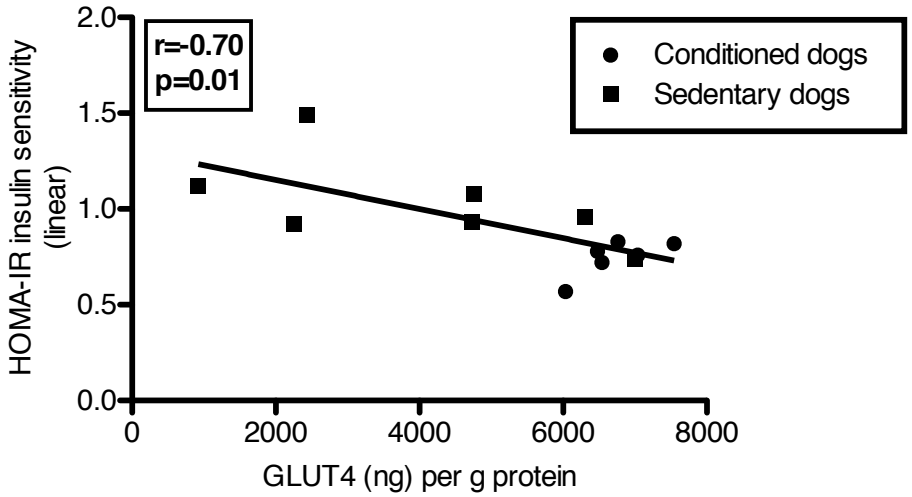

Figure 3.5: The relationship between GLUT4 levels and the homeostasis model assessment of insulin sensitivity HOMA-IR was investigated using Pearson correlation coefficient. HOMA-IR was estimated with the linear model using fasting insulin and glucose levels. Preliminary analysis was performed to ensure no violation of the assumption of normality and linearity. There was moderately close negative correlation between the two variables (r=-0.70, n=13, p=0.01).

Chapter 4

Future Directions

4.1 Refinement of blood sampling protocol

During our study in chapter 2, *Glucose transporter-4 Expression in White Blood Cells of Young and Old Sled Dogs: a model for human biomarker development*, we encountered several difficulties in handling blood samples and extracting the buffy coat (mononuclear interphase layer containing white blood cells). In this first study, blood samples of 4mL were collected in pre-chilled EDTA-treated vacutainer sample tubes containing protease inhibitor cocktail via the cephalic vein. Tubes were stored upright at room temperature for no longer than 2 hours prior to the centrifugation for 15 min at 3600 RPM. The buffy coat was collected and washed in 1x PBS twice. After stimulating with insulin, the mononuclear cells were sonicated, transferred into freezer vials and flash frozen in liquid nitrogen and stored at -80˚C before GLUT4 ELISA analysis.

We attempted buffy coat extraction with a wide variety of micropipettes with limited success. The mononuclear interphase layer turned out being very tiny and fragile and we had to handle it with huge caution, making the process very time consuming. While we were eventually successful, it became apparent that this method would not be a viable option with larger sample sizes. We further had difficulties in breaking up the sticky mononuclear interphase layer and homogenizing the white blood cells when washing in 1x PBS twice without breaking the cells. Unfortunately we were unable to

homogenize the samples without sonification and therefore, in our first attempt we measure total GLUT4 in our samples and not GLUT4 expression on the cell surface.

After encountering those difficulties we refined our blood handling protocol for the study described in chapter 3. Instead of using EDTA Vacutainer sampling tubes we used BD Vacutainer® CPT™ Cell Preparation Tubes containing Sodium Heparin. These tubes are specifically made for the separation of mononuclear cells. Additionally we increased the blood volume from 4mL to 6mL to ensure a bigger mononuclear cell layer volume for easier handling. After centrifugation for 15min at 3600 RPM at RT (same settings as in chapter 2 study), we collected the buffy coat using the following refined protocol: We aspirated half of the blood plasma without disturbing the cell layer. Next, we collected the cell layer with a Pasteur Pipette and transferred the cells into 15mL conical vials. The blood sample was resuspended in 3mL of RPMI w/ 5% calf serum (CS) (instead of 1x PBS as in previous study) and centrifuged for 15 min at 1500 RCF. Mononuclear cells were washed one more time using the previous settings for centrifugation before resuspending in 4mL of RPMI w/ 5% CS.

It turned out that using BD vacutainer cell separation tubes yielded a nice cloud of mononuclear cells that was fairly easy to transfer into 15mL conical vials using a Pasteur Pipette. We are confident that using this method is also more gentle on the cells itself, maintaining cell integrity that ensures the measurement of GLUT4 expression at the cell surface instead of overall GLUT4 content (what we measured in our pilot study in chapter 2 after sonication). Using RPMI w/ 5% CS instead of 1x PBS provided a physiological fluid for washing that kept the samples homogenous and in suspension

after multiple washings and resuspensions, assuring comparable quantities when running the samples in duplicate.

In order to ensure GLUT4 levels were indicative of surface amounts during our study in chapter 3, *Conditioning causes increased GLUT4 levels in mononuclear cells of sled dogs*, we compared levels with samples that were sonicated in chapter 2. Sonicated samples had 3 fold higher GLUT4 levels compared with unsonicated samples. We also measured GLUT4 on the flow cytometer and observed similar trends as a result of exercise [1]. Flow cytometry is the gold standard used in quantifying GLUT4. In addition we adjusted for the amount of protein during our GLUT4 analysis in chapter 3, using a BCA Protein Assay. We are confident that the refinement of our protocol is providing the necessary groundwork for validating the ELISA as a fairly easy, inexpensive, field-friendly and fast method in quantifying GLUT4 expression on mononuclear cells. The ELISA has an added advantage of reporting actual quantities in standard units, where as flow cytometry provides only comparative analysis between groups. It is possible to obtain quantities with a flow cytometer but extensive standardization would be needed. To my best knowledge we were the first group using ELISA instead of flow cytometry to measure GLUT4 levels in mononuclear cells. A next step in validating this technique would be to prepare a subcellular fractionation on samples and quantify GLUT4 in each fraction to ensure that quantification is limited to the cell membrane.

4.2 Exercise protocol

It is well established that exercise training improves insulin action [2-5] and is of critical importance for people with insulin resistance or diabetes [6]. So far, all the work

to understand exercise induced GLUT4 translocation has been made in muscle. Data presented here supports an increase in GLUT4 expression in mononuclear cells as a result of conditioning. In more recent studies, increases in muscle GLUT4 protein concentrations have been detected after short-term exercise in duration of 7-10 days [7] and even more rapidly after just one prolonged exercise session [8]. Another investigation has also shown that GLUT4 protein expression on the cell surface remained elevated for several hours after recovery [9]. These adaptive responses to exercise may contribute to enhanced insulin action in the post-exercise period [10]. Our ongoing research will address the question of whether observations in muscle of increased GLUT4 levels after one acute bout of exercise can also be observed in mononuclear cells.

4.3 Validation in human subjects

Another milestone in developing this technique to provide an innovative, minimally invasive method for assessing insulin sensitivity would be to validate an increase in exercise induced GLUT4 translocation on mononuclear cells in human subjects using ELISA. Based on our findings in sled dogs we would expect that highly conditioned human subjects will have an increased GLUT4 content in mononuclear cells. Correlating mononuclear GLUT4 levels with diet, lifestyle, health indices, physical activity and genetic markers is the goal for our next study using human subjects. Is it possible to use our established blood drawing protocol and commercially available GLUT4 ELISA kits to detect a conditioning response in human subjects as we have seen in sled dogs?

4.4 Clinical application

The standard techniques for the assessment of insulin sensitivity are often either simple indexes that are derived from blood insulin and glucose concentrations under fasting conditions or too invasive for general epidemiologic studies [11, 12]. There is a real need for an easily accessible tissue for routine evaluation of insulin action as alternative to muscle cells. The results presented in chapter 2 and 3 confirm that circulating mononuclear cells, which are easily isolated or sampled from humans, may serve as model system to study the cellular mechanisms of GLUT4 activity, glucose transport and its regulation by insulin and/or exercise. The ability to monitor responses to dietary and exercise interventions in those with insulin resistance and T2D using minimally invasive peripheral blood sampling would have obvious and extensive clinical utility, especially in rural Alaska. Defects in insulin action lead to insulin resistance and T2D. If insulin resistance can be identified prior the development of T2D, there is better hope for prevention by early therapeutic intervention. Future work will focus on establishing the observed exercise responses in mononuclear cells in humans and its correlation to currently used standard techniques for the assessment of insulin sensitivity.

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