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Conditioning causes an increase in Glucose Transporter-4 levels in mononuclear cells in sled dogs

Theresia M. Schnurr^{*,a,b}, Arleigh J. Reynolds^c, Sally J. Gustafson^{a,b}, Lawrence K. Duffy^{a,b}, and Kriya L. Dunlap^{a,b}

^aDepartment of Chemistry and Biochemistry, University of Alaska, Fairbanks, AK 99775, USA

^bInstitute of Arctic Biology, University of Alaska, Fairbanks, AK 99775, USA

^cVeterinary Medical Program, University of Alaska, Fairbanks, AK 99775, USA

Abstract

This study was designed to investigate the effects of physical conditioning on the expression of the insulin sensitive glucose transporter 4 protein (GLUT4) on mononuclear cells and HOMA-IR levels in dogs and compared to results reported in human skeletal muscle and the skeletal muscle of rodent models. Blood was sampled from conditioned dogs (n=8) and sedentary dogs (n=8). The conditioned dogs were exercised four months prior the experiment and were following a uniform training protocol, whereas the sedentary dogs were not. GLUT4 expression in mononuclear cells and plasma insulin levels were measured using commercially available enzyme-linked immunosorbent assay (ELISA). Blood glucose levels were determined using blood plasma. HOMA-IR was calculated using plasma insulin and blood glucose levels using the linear approximation formula. Our results indicate that the state of conditioning had a significant effect on the GLUT4 expression at the surface of mononuclear cells. HOMA-IR was also affected by conditioning in dogs. GLUT4 levels in mononuclear cells of sled dogs were inversely correlated with the homeostasis model assessment of insulin sensitivity. This study demonstrates that conditioning increases GLUT4 levels in mononuclear cells of sled dogs as it has been previously 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.

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***Corresponding author:** Theresia Maria Schnurr, Department of Chemistry and Biochemistry, Institute of Arctic Biology, University of Alaska Fairbanks, 230 West Ridge Research Building, Fairbanks, AK 99775, USA, Tel.: +01 (907) 474-2766, Fax: (907) 474-5640, tmschnurr@gmail.com.

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Keywords

Conditioning; GLUT4; mononuclear cells; insulin sensitivity; exercise

Introduction

The Glucose Transporter-4 (GLUT4) plays 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 (Stöckli et al., 2011). Insulin resistance (IR) 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 type 2 diabetes (T2D) (Bastard et al., 2006). Current methods for diagnosing IR and T2D is often done with a combination of comorbidities and a mathematical index based on fasting glucose-insulin ratios, or glucose tolerance test (Wallace et al., 2004). As prevalence of IR and T2D reach alarming rates (Seidell, 2000, Sharma and Chetty, 2005), the search for direct and reliable diagnostic methods becomes increasingly more important.

GLUT4 is found and studied predominately in muscle and adipose tissue, requiring invasive tissue biopsies (Melling et al., 2013), however as early as 1975, Schwartz et al. reported on insulin binding in monocytes (Schwartz et al., 1975). It was then 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 (Beck-Nielsen et al., 1977). Further, insulin receptors on monocytes have been successfully correlated with glucose intolerance and insulin sensitivity (Beck-Nielsen and Pedersen, 1978). The glucose transporter isoform expressed by subpopulations of mononuclear cells had not been identified until 2002, at which time Korgun et al. discovered the GLUT4 isoform (Korgun et al., 2002). In 2007, Maratou et al. showed an increase in translocation of GLUT4 on the plasma membrane of mononuclear cells collected from human subjects after stimulation with insulin (Maratou et al., 2007). In a subsequent study this research group reported a negative correlation between GLUT4 expression in mononuclear cells and the homeostatic model assessment of insulin resistance (HOMA-IR) in diabetic patients (Maratou et al., 2009). While there is a growing body of research that investigates the presence of GLUT4 expression in mononuclear cells and the effects of insulin, to our knowledge no research has been performed on exercise induced GLUT4 translocation in mononuclear cells.

Regular exercise has a wide array of health promoting effects and much research is directed at better understand the molecular mechanisms underlying these benefits (Carey and Kingwell, 2009). Exercise training has been shown to mediate skeletal muscle enzymes, transcription factors, transporters and chaperones through an adaptive response to chronic training (Carey and Kingwell, 2009). Of particular interest for this study are well-documented effects of exercise on GLUT4 in skeletal muscle. The exercise-induced response occurs by recruiting more GLUT4 to the cell surface from a larger total muscle pool of GLUT4 (Reynolds et al., 2000) and the resulting increase in muscle GLUT4 protein is associated with an increased capacity for glucose transport (Rodnick et al., 1992,

Goodpaster et al., 2001). Similar findings have been reported in rats (Ploug et al., 1990). 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 (Host et al., 1998, Ren et al., 1994).

Sled dogs are elite athletes whose energy expenditure and physical endurance provide for an excellent model for studying the effects of exercise on insulin signaling and glucose uptake (Hinchcliff et al., 1997a). 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 studying human metabolic disorders for over a century (Catchpole et al., 2005, Serisier et al., 2008). Though there are species-specific pathologies associated with diabetes, dogs develop insulin dependent and independent forms of diabetes, and gestational diabetes akin to humans (Catchpole et al., 2005, Bergman et al., 2006, Johnson, 2008). While the prevalence of canine diabetes remains lower than humans, an increasing trend in dogs has been observed (Catchpole et al., 2005). Furthermore, the most widely used clinical and epidemiological tool for assessing insulin sensitivity, the homeostatic model assessment, HOMA-IR (Wallace et al., 2004) has also been established in the dog (Serisier et al., 2008).

We report, for the first time, that GLUT4 levels in mononuclear cells are elevated in response to conditioning. Furthermore, GLUT4 in mononuclear cells in sled dogs displayed a negative correlation with HOMA-IR. These results provide additional support in the growing body of evidence that mononuclear cells are a promising proxy tissue for assessing insulin sensitivity.

Methods

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 exercise program for the conditioned sled dogs over the 4 months prior the blood draw (May- September) consisted of varying modalities, durations, and intensities. The goal of the training program is to slowly increase miles and speed. Since this was during the summer, the training program was primarily for maintenance, which consists mostly of hooking sled dogs pulling in harness in front of an All Terrain Vehicle (ATV) and maintaining an approximate speed of between 15-18 mph for 3-8 miles. This is interspersed with long duration (2-3 hours), low intensity exercise (approximately 7mph), that involves an exercise wheel. An exercise wheel is a bi-directional motorized wheel that is commonly used for exercising horses and has been adapted for dogs. The animals are similarly fastened to the wheel by chains that hang down off of large extended arms to their collars and a fast walk is maintained without resistance. 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). We compared

GLUT4 concentrations between males and females in both groups to ensure that sex was not a confounding issue (t-test, $p>0.05$). 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 (Laflamme, 1997).

Blood sampling

The temperature range on the day of the experiment was 4-7°C (September 5th, 2012). 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 r.p.m 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 enzyme-linked immunosorbent assay (ELISA) analysis and adjusted for protein content using BCA Protein assay.

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 2-3 fold higher GLUT4 levels compared with unsonicated samples (Schnurr et al., 2013). A BCA Protein Assay (Pierce, Thermo Scientific, United States) was used for protein adjustment in regard with GLUT4 and absorbance was read at 562 nm. Insulin levels were measured using an 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 $\mu\text{U/mL}$) and glucose (in mmol/L) concentrations by 22.5 (Matthews et al., 1985). All samples were assayed in duplicates as an internal control.

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 well below 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 conditioning 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 $p < 0.05$. All data are reported as means $\pm$ s.d.

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 remained sedentary (Fig. 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). Conditioned dogs also displayed less variability when compared to the sedentary dogs (Fig. 1).

Plasma Insulin concentrations

Plasma insulin concentrations did not significantly differ between sedentary dogs (3.8 ± 0.69 μ U/mL) when compared to the conditioned dogs (3.3 ± 0.23 μ U/mL, Fig. 2, $p > 0.05$).

Plasma Glucose concentrations

Sedentary dogs displayed significantly higher glucose levels (110 ± 3 mg/mL) when compared to the conditioned group (96 ± 5 mg/mL, Fig. 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. 4). HOMA-IR was calculated for each individual dog using fasting insulin and glucose levels in a resting state, prior to any exercise.

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. 5). Sedentary dogs showed higher HOMA-IR and lower GLUT4 levels on the plasma membrane of mononuclear cells compared to conditioned dogs.

Discussion and Conclusions

We report, for what we believe is the first time, an effect of conditioning on GLUT4 levels in mononuclear cells (Fig.1). This agrees with previous reports in muscle, demonstrating that an increase in GLUT4 protein expression on the cells surface is an important chronic

adaptation to exercise training, parallel to an increase in mitochondria and oxidative capacities and the transformation of muscle fiber types (Rockl et al., 2008) (Assali et al., 2001). 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 (Sun et al., 2013). Our results also suggest that four months of exercise training lead to improved insulin sensitivity as indicated by a lower HOMA-IR (Fig. 4).

Most often GLUT4 in mononuclear cells is measured using flow cytometry after stimulating the cells with insulin (Maratou et al., 2007). This study evaluated GLUT4 expression on mononuclear cells without insulin stimulation using a commercially available ELISA. This approach was selected to provide a simple, inexpensive and reproducible technique for reporting GLUT4 concentrations, rather than arbitrary units, and at the same time eliminate the need for technical and expensive equipment. We have validated this approach in a couple of ways. Initially we compared sonicated samples with samples that were left intact and as expected sonicated samples were 2-3 fold higher in GLUT4. A study is in progress in which we are looking at the effects of one acute bout of exercise on GLUT4 in mononuclear cells of sled dogs in which all sample have been run on both an ELISA and flow cytometer to further validate the use of ELISA. Results, thus far, are supportive.

While dogs are a good model for obesity (Serisier et al., 2008), this study suggests that sled dogs are an excellent model for the effect of exercise on energy metabolism. Exercise and conditioning has a well-established effect on insulin sensitivity and GLUT4 expression (Ebeling et al., 1993) and 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 (Hinchcliff et al., 1997b). Sled dogs are incredible athletes that provide a homogenous population for studying environmental impacts such as nutrition and exercise on blood parameters (Reynolds et al., 1997, Reynolds et al., 1999, Dunlap et al., 2006). It should be emphasized that even the sedentary sled dogs in this study are relatively fit compared with other canine models (Dunlap et al., 2006) and 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 raced 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 is further 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 compared with unaffected populations.

Another marker for insulin resistance and T2D that is becoming increasingly popular in the clinical setting is glycosylated hemoglobin (HbA1c) (Jansink et al., 2013). 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 (Wallace et al., 2004), but the assumptions used in HOMA-IR analysis have shown to largely hold true in dogs (Verkest et al., 2010).

The clinical implication of both insulin and exercise GLUT4 amplification in mononuclear cells is an exciting new research area. Our results support the extensive body of research in skeletal muscle and substantiate evidence that mononuclear cells have the potential to provide an excellent proxy tissue for assessing GLUT4 with a simple, direct and low-invasive technique that has the potential to serve as a diagnostic tool and would likely enhance nutritional and exercise intervention studies (Ploug et al., 1990, McLeay et al., 2012, Reynolds et al., 1997). We have several on-going projects that will further our understanding of exercise on GLUT4 response in mononuclear cells. This includes, as previously mentioned, evaluating the effects acute exercise on GLUT4 in sled dogs, and most recently, we are applying our methods in human student athletes.

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List of Abbreviations

BCA	Bicinchoninic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
GLUT4	Glucose Transporter 4 protein
HbA1c	Glycated hemoglobin
HOMA-IR	Homeostasis Model assessment of insulin resistance
IACUC	The Institute of Animal Care and Use Committee
IR	Insulin resistance
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
T2D	Type 2 diabetes
WBC	White blood cells

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Figure 1. GLUT4 concentrations on the surface of mononuclear cells of sled dogs is effected by long-term conditioning

GLUT4 concentrations were measured via enzyme linked immunoassay 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 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 $3.3 \pm 0.23 \mu\text{U/mL}$ and in sedentary dogs $3.8 \pm 0.69 \mu\text{U/mL}$. Although data show an apparent trend, means were not significantly different (t-test, $p > 0.05$).

Figure 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 4. The Homeostasis Model assessment to quantify insulin resistance (HOMA-IR) is significantly lower in conditioned dogs 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 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 (●=conditioned dogs, □=sedentary dogs, $r=-0.70$, $n=13$, $p=0.01$).