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MUSCLE GLYCOGEN CONCENTRATIONS, GLUT4 AND MUSCLE DAMAGE IN HUMANS FOLLOWING ECCENTRIC EXERCISE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Scott Carl Swanson, B.S., M.S.

* * * * *

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1996

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ABSTRACT

Initial Experiment

Muscle glycogen concentrations are reduced 24-72 h after eccentric exercise that produces muscle damage in humans. However, the effects of eccentric exercise in the rat are unclear. Thus, an initial study was conducted to study the effects of eccentric exercise on muscle glycogen concentrations and morphology in the rat. A total of 16 male Sprague-Dawley rats underwent percutaneous muscle stimulation of the plantar flexors. One hindlimb underwent concentric (CON) contractions while the contralateral hindlimb underwent a combination of concentric and eccentric (CON/ECC) contractions. Muscles of the lower hindlimb were removed immediately after and 1, 2, 3, an 5 d after the contraction protocol. The extent of possible muscle damage was also assessed by electron microscopy 3 d after the contraction protocol. Compared to pre-contraction, muscle glycogen concentrations were reduced immediately after exercise in both the CON and CON/ECC leg and returned to normal within 1 d after the contraction protocol. There were no differences in CON and CON/ECC muscle glycogen
concentrations at 1, 2, 3, or 5 d after the contraction protocol. In addition, evidence of muscle damage in the CON and CON/ECC leg were similar 3 d after the contraction protocol. It was concluded that eccentric contractions produced by percutaneous muscle stimulation of the plantar flexors in the rat does not reduce muscle glycogen concentrations nor produce muscle damage to a greater extent than concentric contractions.

**Main Experiment**

Because eccentric contractions produced by percutaneous muscle stimulation in the rat did not reduce muscle glycogen concentrations or produce muscle damage, another experiment was conducted in humans to examine muscle glycogen concentrations after eccentric exercise. The rate of muscle glycogen synthesis and skeletal muscle glucose uptake are regulated by the GLUT4 isoform. GLUT4 is translocated from an intracellular storage site to the plasma membrane, where it increases glucose transport. Eccentric exercise damages the plasma membrane and disrupts the ultrastructure of skeletal muscle. Thus, muscle damage may decrease the concentration of GLUT4 in the plasma membrane, thereby reducing glucose transport and muscle glycogen concentrations.

A total of 12 human subjects participated in this study. Subjects performed concentric (CON) knee extensions
with one leg while the contralateral leg performed eccentric (ECC) knee extensions. Subjects then cycled for 1 h to reduce muscle glycogen concentrations. Seventy-two h later, subjects then underwent another glycogen-depleting ride and subsequently consumed a carbohydrate beverage every 15 min for 4 h of recovery. Muscle biopsies were obtained from each leg 72 h after the initial exercise session (PRE-EX), immediately after the second glycogen-depleting ride (POST-EX), and after the carbohydrate feeding period (POST-CHO). Biopsies were analyzed for muscle glycogen and total GLUT4 protein concentrations. Skeletal muscle GLUT4 transporters were documented by immunocytochemistry, and muscle damage was assessed by electron microscopy.

Compared to the CON leg, PRE-EX muscle glycogen concentrations were ~25% lower in the ECC leg (p<0.05). However, CON and ECC muscle glycogen concentrations were not different at POST-EX and POST-CHO. CON and ECC total GLUT4 concentrations were similar at all measured times. Muscle damage such as myofibrillar disruptions and necrotic fibers were apparent in the ECC leg 72 h after the initial exercise session, whereas the CON leg did not show any signs of damage. Localization of GLUT4 by immunocytochemistry in both the CON and ECC leg were not altered 72 h after the initial exercise session.
Thus, muscle glycogen concentrations were reduced and muscle damage was produced in the ECC leg 72 h after eccentric exercise in humans. In addition, muscle GLUT4 protein concentrations and GLUT4 localization by immunocytochemistry were not altered 72 h after the initial exercise session.
To My Parents,

For all the love and support they have given me throughout my academic career.
ACKNOWLEDGEMENTS

To my parents, I offer sincere thanks for the encouragement and support you have given me in all endeavors through my life.

I express sincere appreciation to my advisor, Dr. W. Michael Sherman, for his assistance, insight and guidance throughout my doctoral studies at The Ohio State University. Thanks also go to the other members of my advisory committee, Drs. David R. Lamb and Richard W. Burry, for their comments and suggestions. The technical assistance of Kathy Wolken in the Campus Microscopy and Imaging Facility is gratefully acknowledged.

Many individuals helped with this project and I would particularly like to thank Dr. Greg Wimer, Brian Bettendorf, Chris Weising and Kris Engstrom and the other graduate and undergraduate students that assisted. My gratitude also goes to the subjects who volunteered and took part in this project.

I also acknowledge Dr. Mitch Kanter and The Quaker Oats Company for their generous support.
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**Major Field:** Health, Physical Education and Recreation

(Exercise Physiology)
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Muscle glycogen is reduced after eccentric exercise, but the mechanism(s) for the decrease in muscle glycogen is(are) unknown. The rate of muscle glycogen synthesis is limited by the rate of glucose transport into the muscle cell. Skeletal muscle glucose transport occurs via a facilitated process that is regulated by the glucose transporter GLUT4. After contraction or insulin-stimulation, GLUT4 is translocated from an intracellular pool to the plasma membrane where it increases glucose transport.

Eccentric exercise produces muscle damage, e.g., myofibrillar disruptions, plasma membrane damage and muscle fiber necrosis, that may decrease skeletal muscle GLUT4 and glucose transport. A decrease in glucose transport after eccentric exercise may reduce the muscle glycogen concentration. The overall purpose of this dissertation is to determine the relationships among muscle damage, muscle glycogen concentrations, and GLUT4 after eccentric exercise.
Initial Experiment

The intramuscular responses to eccentric exercise are thought to be similar in both humans and rats. In fact, no literature exists that suggests differences between rat and human muscle in response to eccentric exercise. After sacrifice, large amounts of muscle tissue (i.e., 1-3 g) can be collected and analyzed in rats. In contrast, the amount of muscle tissue that is obtained via muscle biopsies in man is much smaller (30-120 mg). Therefore, biochemical and histochemical analyses are not typically limited by the amount of skeletal muscle tissue available from rats. An attempt was made during this initial study to design an exercise device in rats that would produce muscle damage after eccentric exercise. Therefore, an initial study was undertaken to examine muscle glycogen concentrations and muscle damage after eccentric exercise in rats.

For the initial study, rats underwent percutaneous muscle stimulation of the hindlimb plantar flexors. One leg underwent concentric contractions whereas the contralateral hindlimb underwent a combination of eccentric and concentric contractions. Muscles of the plantar flexors were removed immediately after and 1, 2, 3, and 5 d after the contraction protocol. Muscle glycogen was reduced immediately after contractions in both legs. Muscle glycogen was not reduced after eccentric contractions at any measured time during
recovery. In addition, the contraction protocol did not produce muscle damage.

**Main Experiment**

Because the initial study in rats did not reduce muscle glycogen after eccentric exercise or produce muscle damage, a second study was performed in humans. Muscle glycogen is reduced 24-72 h after eccentric exercise that produces muscle damage in humans. In addition, GLUT4 is decreased after eccentric exercise. Thus, a second experiment was conducted to examine muscle GLUT4 and glycogen concentrations 72 h after eccentric exercise that produces muscle damage in humans.

Subjects in the human study performed concentric knee extensions with one leg whereas the contralateral leg performed eccentric knee extensions. Subjects then cycled on an ergometer to deplete muscle glycogen concentrations in both legs. Seventy-two hours later, subjects performed a second session of cycling exercise and consumed a carbohydrate beverage during the initial 4 h of recovery to provide a substrate for muscle glycogen synthesis. Muscle biopsies were obtained from each leg 72 h after the initial exercise session, after the second cycling bout, and after the 4 h carbohydrate feeding period. Biopsies were analyzed for muscle glycogen and total GLUT4 concentrations. Muscle GLUT4 transporters were documented by immunocytochemistry.
Muscle damage was documented by electron microscopy.
Muscle glycogen concentrations are reduced after eccentric exercise that produce muscle damage in man. It is uncertain if muscle glycogen concentrations are reduced after eccentric exercise that produces muscle damage in the rat. This study examined muscle glycogen concentrations after eccentric muscle contractions in the rat. Sixteen rats underwent percutaneous muscle stimulation of the plantar flexors. One hindlimb underwent concentric contractions (CON) whereas the contralateral hindlimb underwent a combination of concentric and eccentric muscle contractions (CON/ECC). Rats (n=4 per group) were sacrificed immediately after and at 1 d, 2 d, 3 d, and 5 d after contractions. Soleus (SOL), plantaris (PLA), gastrocnemius (GAS), extensor digitorum longus (EDL) and tibialis anterior (TA) were excised, analyzed for muscle glycogen concentrations, and processed for electron microscopy. Post-contraction CON and CON/ECC muscle glycogen concentrations were significantly depleted to
similar levels in all muscles except for CON/ECC SOL, CON EDL and CON/ECC EDL. CON and CON/ECC muscle glycogen concentrations returned to pre-exercise levels by 1 d and were similar for up to 5 d after contraction. Muscle damage was not produced after eccentric contractions of the SOL and PLA. In addition, muscle glycogen concentrations were similar for up to 4 d after 500 eccentric or concentric muscle contractions. In summary, percutaneous muscle stimulation did not reduce muscle glycogen concentrations for up to 5 d or produce muscle damage after eccentric muscle contractions of the plantar flexors in the rat.
Muscle Glycogen Concentrations and Muscle Morphology in the Rat Following Eccentric Exercise

During eccentric muscle contractions, muscle fibers lengthen while tension is developed (e.g., lowering a weight). Muscle glycogen concentrations are reduced after eccentric exercise that produces muscle damage in humans, but the effect of eccentric contractions on muscle glycogen concentrations in the rat is unclear. For example, Ferry et al. (1992) reported that muscle glycogen synthesis in the rat was not altered for up to 52 h after downhill (eccentric) running. However, no indices of muscle damage were reported, and drug interventions and post-exercise starvation for 48 h after exercise confound interpretation of their results. Also, Van der Meulen et al. (1992) reported that muscle glycogen concentrations in the rat were similar up to 24 h after electrically-stimulated isometric or lengthening (eccentric) muscle contractions. However, Asp et al. (1995b) found that muscle glycogen concentrations were decreased 24 h and 48 h after electrically-stimulated eccentric contractions of the lower hindlimb muscles in the
rat. Thus, there is no consensus on whether or not eccentric muscle contractions reduce muscle glycogen concentrations in the rat.

Protocols for producing muscle damage after eccentric exercise in humans are easily accomplished because humans will voluntarily perform eccentric muscle contractions. However, the amount of muscle tissue obtained via muscle biopsies is small (e.g., 30-75 mg) and may limit subsequent biochemical and histochemical analyses. Eccentric exercise protocols in rats are somewhat more difficult to design and have included downhill running (Armstrong et al., 1983; Ogilvie et al., 1988) and electrical muscle stimulation (Asp et al., 1995b; Van der Meulen et al., 1992; Wong & Booth, 1988; Kirby et al., 1992). Downhill running protocols damage only the soleus (Ogilvie et al., 1988), and the mass of the soleus is small (i.e., 50-100 mg). However, eccentric exercise protocols employing electrical muscle stimulation can be designed to stimulate a variety of muscles, and the amount of muscle tissue that can be obtained (i.e., 1-3 g) is relatively large. Therefore, muscle tissue obtained from electrical muscle stimulation protocols in rats will not limit subsequent biochemical and histochemical analyses.
Studies reporting reduced muscle glycogen concentrations after eccentric exercise in humans have documented muscle damage with both light and electron microscopy (Frieden et al., 1983; Hikida et al., 1983). Unfortunately, studies investigating muscle glycogen concentrations after eccentric exercise in the rat have assessed muscle damage by relatively indirect indices of muscle damage such as leukocyte/phagocyte infiltration, focal muscle necrosis and inflammatory changes (Asp et al., 1995b; Van der Meulen et al., 1992; Ferry et al., 1992). Therefore, it is uncertain if actual muscle damage produced by eccentric contractions is associated with altered muscle glycogen concentrations in the rat. Nonetheless, it would not be anticipated that there would be differences in the responses to eccentric exercise in rats and humans.

**Purpose**

An initial study was undertaken to examine muscle glycogen concentrations after eccentric muscle contractions in rats. These contractions were produced by an eccentric exercise device designed to produce muscle damage in the lower hindlimb muscles. Rats underwent eccentric and concentric plantar flexions via percutaneous electrical stimulation of the lower hindlimb muscles. Muscles were removed and analyzed for muscle glycogen concentrations and
muscle damage.

**Study Design**

A total of 16 rats (n = 4 per group) underwent concentric and/or eccentric plantar flexion by percutaneous electrical muscle stimulation. The rear foot of each rat was strapped to a footplate device designed for plantar flexion (Wong & Booth, 1988) (see Appendix F for schematic), and needle electrodes were inserted percutaneously below the knee and at the Achilles tendon. A concentric one-repetition maximum (1 RM) was first determined for each hindlimb. The 1 RM was defined as the maximum weight each hindlimb could plantar flex during continuous maximal stimulation.

One randomly assigned hindlimb (CON) underwent 100 concentric plantar flexions at 70% of the concentric 1 RM plus an additional six sets of 12 concentric plantar flexions at 50% of the concentric 1 RM. The contralateral hindlimb (CON/ECC) underwent 100 concentric plantar flexions at 70% of the concentric 1 RM plus an additional six sets of eight eccentric plantar flexions at 120-150% of the concentric 1 RM. Time between sets was 2 min. Rats were stimulated at 12-15 V, a frequency of 60 pps and a duration of 500 ms. Thus, one hindlimb (CON) underwent only concentric plantar flexions, whereas the contralateral hindlimb (CON/ECC) underwent a combination of concentric and
eccentric plantar flexions. Total work (positive + negative) was similar for both hindlimbs.

Rats were sacrificed immediately after and 1, 2, 3, and 5 d after the contraction protocol. The soleus (SOL), gastrocnemius (GAS), plantaris (PLA), extensor digitorium longus (EDL) and tibialis anterior (TA) were removed and stored at -80 °C until analysis. Muscle was analyzed for muscle glycogen concentrations (Passoneau and Lauderdale, 1974) and processed for ultrastructural analysis by electron microscopy (Appendix G).

Statistics

An ANOVA was used to analyze mean differences in muscle glycogen concentrations between the hindlimbs at each measured time.

Results

Compared to pre-contraction muscle glycogen concentrations, post-contraction muscle glycogen concentrations were decreased in all muscles (p<0.05) except for CON EDL, CON/ECC EDL and CON/ECC SOL (Table 1). Muscle glycogen concentrations were reduced to similar levels in the other CON and CON/ECC hindlimb muscles.

Muscle glycogen concentrations returned to the pre-contraction concentrations within 1 d after the contraction protocol, and there were no significant differences in muscle glycogen concentrations between CON and CON/ECC
Table 1. Muscle glycogen concentrations (mmol glu·kg wet wt⁻¹) of CON and CON/ECC hindlimbs immediately after the contraction protocol.

<table>
<thead>
<tr>
<th>MUSCLE</th>
<th>Pre-ex</th>
<th>Post-ex CON</th>
<th>Post-ex CON/ECC</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOL</td>
<td>34.4 ±3.32</td>
<td>*25.7 ±1.50</td>
<td>30.6 ±1.37</td>
</tr>
<tr>
<td>PLA</td>
<td>39.2 ±3.61</td>
<td>*20.9 ±0.86</td>
<td>*24.4 ±3.40</td>
</tr>
<tr>
<td>GAS</td>
<td>43.3 ±3.50</td>
<td>*21.2 ±2.23</td>
<td>*20.8 ±1.37</td>
</tr>
<tr>
<td>EDL</td>
<td>33.3 ±3.31</td>
<td>26.8 ±3.43</td>
<td>25.7 ±3.73</td>
</tr>
<tr>
<td>TA</td>
<td>38.8 ±2.81</td>
<td>*17.4 ±1.92</td>
<td>*18.6 ±1.51</td>
</tr>
</tbody>
</table>

Values are expressed as Means ±SEM; * = Significantly different (p<0.05) than pre-ex; SOL=soleus; PLA=plantaris; GAS=gastrocnemius; EDL=extensor digitorium longus; TA=tibialis anterior.
hindlimbs at 1, 2, 3, or 5 d after the contraction protocol (Table 2). In addition, CON and CON/ECC muscle ultrastructure was similar 3 d after the contraction protocol (Figures 4 and 5). Thus, the contraction protocol produced no evidence of selective muscle damage or reduced muscle glycogen associated with eccentric contractions.

In an attempt to cause moderate muscle damage after eccentric contractions, an additional six rats (n = 3 per group) underwent only concentric or eccentric plantar flexions with each hindlimb. In this case, the number of contractions was doubled to 200 per contraction type, and rats were sacrificed 2 d after contractions. The CON leg initially began plantar flexions at 70% of the concentric 1 RM, and the load was progressively reduced to 30-40% of the concentric 1 RM to successfully complete the 200 contractions. The ECC leg underwent 200 contractions at 120% of the concentric 1 RM. In an attempt to further induce muscle damage, another four rats (n = 2 per group) performed 500 concentric or 500 eccentric plantar flexions with each hindlimb. The CON leg initially began plantar flexions at 70% of the concentric 1 RM and the load was progressively reduced to 20% of the concentric 1 RM to allow successful completion of the 200 contractions. These rats were sacrificed 2 d and 4 d after contractions. The muscles of the lower hindlimbs of both groups were removed and
Table 2. Muscle glycogen concentrations (mmol glu·kg wet wt⁻¹) of CON and ECC hindlimbs 1, 2, 3, and 5 d after contraction protocol.

<table>
<thead>
<tr>
<th>MUSCLES</th>
<th>DAY 1</th>
<th>DAY 2</th>
<th>DAY 3</th>
<th>DAY 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON</td>
<td>ECC</td>
<td>CON</td>
<td>ECC</td>
</tr>
<tr>
<td>SOL</td>
<td>29.0  ±3.64</td>
<td>28.6 ±2.39</td>
<td>30.3  ±2.52</td>
<td>30.1  ±3.26</td>
</tr>
<tr>
<td>PLA</td>
<td>29.7  ±1.55</td>
<td>31.2 ±1.98</td>
<td>32.9  ±1.28</td>
<td>29.1  ±1.94</td>
</tr>
<tr>
<td>GAS</td>
<td>57.0  ±9.75</td>
<td>48.3 ±5.31</td>
<td>37.9  ±1.58</td>
<td>34.4  ±1.074</td>
</tr>
<tr>
<td>EDL</td>
<td>26.3  ±1.55</td>
<td>25.6 ±2.56</td>
<td>29.2  ±0.58</td>
<td>26.4  ±2.79</td>
</tr>
<tr>
<td>TA</td>
<td>25.1  ±2.00</td>
<td>23.4 ±3.06</td>
<td>35.1  ±2.43</td>
<td>29.1  ±1.57</td>
</tr>
</tbody>
</table>

Values are expressed as Means ±SEM; SOL=soleus; PLA=plantaris; GAS=gastrocnemius; EDL=extensor digitorium longus; TA=tibialis anterior.
Figure 1. Muscle ultrastructure of rat plantaris muscle 3 d after concentric contractions (7500X).
Figure 2. Muscle ultrastructure of rat plantaris muscle 3 d after concentric and eccentric contraction protocol (9500X)
analyzed for muscle glycogen concentrations (Passoneau and Lauderdale, 1974).

Muscle glycogen concentrations were similar 2 d after 200 concentric or 200 eccentric plantar flexions. Likewise, muscle glycogen concentrations were similar 2 d and 4 d after 500 concentric or 500 eccentric plantar flexions (Table 3).

Conclusions

It was concluded that eccentric contractions induced by percutaneous electrical muscle stimulation did not produce lower muscle glycogen concentrations when compared to concentric contractions in the rat. This conclusion held for a wide number of protocols, including up to 500 eccentric-only contractions. Therefore, this model of concentric or eccentric contractions in the rat was not useful to study the effects of eccentric contractions on muscle damage and muscle glycogen concentrations.
Table 3. Muscle glycogen concentrations (mmol glu·kg wet wt⁻¹) 2 d after 200 concentric (D2 200CON), 500 concentric (D2 500CON), 200 eccentric (D2 200ECC) or 500 eccentric (D2 500ECC) contractions and 4 d after 500 concentric (D4 500CON) or 500 eccentric (D4 500ECC) contractions.

<table>
<thead>
<tr>
<th>Muscle</th>
<th>D2 200CON</th>
<th>D2 200ECC</th>
<th>D2 500CON</th>
<th>D2 500ECC</th>
<th>D4 500CON</th>
<th>D4 500ECC</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOL</td>
<td>32.2 ±4.31</td>
<td>32.8 ±3.05</td>
<td>30.2 ±4.95</td>
<td>28.2 ±3.62</td>
<td>25.0 ±1.55</td>
<td>25.2 ±0.80</td>
</tr>
<tr>
<td>PLA</td>
<td>30.3 ±2.01</td>
<td>31.9 ±2.39</td>
<td>27.5 ±0.05</td>
<td>27.2 ±1.40</td>
<td>23.4 ±2.75</td>
<td>26.5 ±5.90</td>
</tr>
<tr>
<td>GAS</td>
<td>32.4 ±1.40</td>
<td>31.7 ±0.70</td>
<td>24.8 ±0.50</td>
<td>27.2 ±1.45</td>
<td>18.5 ±0.95</td>
<td>24.1 ±4.20</td>
</tr>
<tr>
<td>EDL</td>
<td>28.1 ±1.92</td>
<td>28.8 ±2.33</td>
<td>21.2 ±0.30</td>
<td>23.6 ±0.60</td>
<td>23.3 ±6.10</td>
<td>26.5 ±2.20</td>
</tr>
<tr>
<td>TA</td>
<td>32.4 ±2.06</td>
<td>38.9 ±1.91</td>
<td>26.3 ±0.20</td>
<td>28.1 ±0.35</td>
<td>23.8 ±4.85</td>
<td>29.5 ±7.15</td>
</tr>
</tbody>
</table>

Values are Mean ±SEM; SOL = soleus; PLA = plantaris; GAS = gastrocnemius; EDL = extensor digitorium longus; TA = tibialis anterior.
CHAPTER II

Muscle Glycogen Concentrations, GLUT4 and Muscle Damage in Humans Following Eccentric Exercise

Abstract

Muscle glycogen concentrations are reduced 24-72 h after eccentric exercise that produces muscle damage in humans. However, the mechanism responsible for the reduced muscle glycogen is unknown. The rate of skeletal muscle glucose uptake is regulated by glucose transport, which is regulated by the GLUT4 isoform. GLUT4 is translocated from an intracellular storage site to the plasma membrane, where it increases glucose transport. Eccentric exercise damages the plasma membrane and disrupts the ultrastructure of skeletal muscle. Thus, muscle damage may decrease GLUT4 concentrations in the plasma membrane. This may reduce glucose transport and muscle glycogen after eccentric exercise. The purpose of this study was to examine muscle glycogen and GLUT4 concentrations after exercise that produces muscle damage in humans.

A total of 12 college-aged individuals participated in
this study. Subjects initially performed 5 sets of 10
concentric knee extensions (CON) at 70% of the concentric 1
repetition maximum (1 RM) with one leg. The contralateral
leg performed 10 sets of 10 eccentric knee extensions (ECC)
at 120% of the concentric 1 RM. Subjects then cycled for 1
h at 65% VO$_{2\text{max}}$ to lower muscle glycogen concentrations.
Seventy-two h later, subjects then underwent another
glycogen-depleting ride and subsequently consumed a 0.4 g
CHO·kg BW$^{-1}$ every 15 min for 4 h of recovery. Muscle
biopsies were obtained from each leg 72 h after the initial
exercise session (PRE-EX), immediately after the second
glycogen-depleting ride (POST-EX), and immediately after the
4 h carbohydrate consumption period (POST-CHO). Biopsies
were analyzed for muscle glycogen and total GLUT4 protein
concentrations. Skeletal muscle GLUT4 was observed by
immunocytochemistry. Muscle damage was assessed by electron
microscopy.

Compared to the CON leg, PRE-EX muscle glycogen
concentrations in the ECC leg were ~25% lower (p<0.05).
However, CON and ECC muscle glycogen concentrations were not
different at POST-EX and POST-CHO. There was no difference
between CON and ECC total GLUT4 protein concentrations at
any time. In addition, total GLUT4 protein concentrations
were similar at all measured times. Muscle damage such as
myofibrillar disruptions and necrotic fibers were apparent
in the ECC leg, whereas the CON leg did not show signs of muscle damage. Thus, muscle glycogen concentrations were reduced, and muscle damage was produced in the ECC leg 72 h after exercise. Muscle GLUT4 protein concentrations and of GLUT4 localization by immunocytochemistry in both the CON and ECC leg was not altered 72 h after the initial exercise session.
Introduction

Muscle glycogen concentrations are reduced 24-72 h after eccentric exercise that produces muscle damage in humans, but the mechanism for the decrease in muscle glycogen is unknown. Thus, an experiment was undertaken to investigate a possible mechanism for the reduced muscle glycogen concentrations observed after eccentric exercise in humans.

In humans, the rate of glucose uptake into the cell limits the rate of muscle glycogen synthesis (Freidman et al., 1991). Glucose uptake and transport occurs via a facilitated process that is regulated by a family of glucose transporters. The primary glucose transporters in mammalian skeletal muscle are GLUT1 and GLUT4 (Klip and Paquet, 1990; Kahn, 1992). GLUT1 is found in low concentrations in muscle tissue and regulates basal glucose uptake. GLUT4 is found in abundant concentrations in skeletal muscle and is responsible for insulin- and contraction-stimulated skeletal muscle glucose uptake (Klip and Paquet, 1990; Kahn, 1992).

GLUT4 transporters are located intracellularly in or near triads and transverse tubules, subsarcolemma vesicles
and Golgi-like vesicles at rest (Friedman et al., 1991; Borneman et al., 1992). The intracellular site(s) for glucose transporters may be disrupted after eccentric exercise, and this may be related to the observed reductions in muscle glycogen concentrations after eccentric exercise. Eccentric exercise causes extensive damage to the subsarcolemma and intracellular structures of muscle fibers, including A-band and Z-line streaming, mitochondrial swelling, and in severe cases, total fiber disruption and necrosis of the affected fibers. In the latter stages of the responses to eccentric exercise, focal disruptions such as A-band and Z-line streaming can affect more than 50% of the muscle fibers (Frieden et al., 1983).

Because the transverse tubules are located at the A-I band junctions, A-band streaming may damage the storage site for GLUT4 transporters. In addition, extensive Z-line streaming may further compromise the integrity of transverse tubules. Intracellular disruptions can also affect the entire width of a fiber, possibly damaging vesicles and the Golgi-apparatus that also contain GLUT4 transporters. Thus, the two primary storage sites for GLUT4 transporters may be disrupted after eccentric exercise.

Plasma membrane damage also occurs after eccentric exercise damage (Hikida et al., 1983; McNeil and Khakee,
1992), and this may decrease the number of glucose transporters incorporated into the plasma membrane. Damage to the plasma membrane after eccentric exercise may decrease the amount of GLUT4 transporters in the plasma membrane, thereby decreasing the transport of glucose into the cell. Thus, the amount of glucose available for glycogen synthesis may be decreased by plasma membrane damage after eccentric exercise.

Asp et al. (1995a) demonstrated that the total GLUT4 concentration in the crude muscle membrane and muscle glycogen concentration are significantly lower 24 h and 48 h after eccentric exercise. It was suggested that the plasma membrane damage associated with eccentric exercise may cause defective glucose transporter translocation or a more rapid degradation of GLUT4 compared to the synthesis of GLUT4 (Asp et al., 1995a). A decrease in the muscle GLUT4 protein concentration after eccentric exercise should decrease glucose transport into the muscle cell and reduce the amount of glucose available for muscle glycogen synthesis.

It was hypothesized that muscle glycogen concentrations and the total number of GLUT4 transporters in the plasma membrane of eccentrically damaged muscle will be decreased 3 d after eccentric exercise. This decreased number of GLUT4 transporters in the plasma membrane in response to insulin
stimulation may help explain the reduced muscle glycogen concentrations (Widrick et al., 1992; Costill et al., 1990; Asp et al., 1995a) and the decreased rate of muscle glycogen synthesis (Doyle et al., 1993) observed 24-72 h after eccentric exercise.

The general purposes of this study were:

1) to determine muscle glycogen concentrations after eccentric exercise in humans;

2) to document muscle damage after eccentric exercise in humans;

3) to characterize muscle GLUT4 after eccentric exercise in humans.
Eccentric Exercise and Muscle Glycogen Synthesis

Muscle glycogen concentrations typically return to "normal" 24 h after concentric exercise (Bergstrom and Hultman, 1966; Bergstrom et al., 1972; Costill et al., 1981). However, muscle glycogen concentrations are significantly reduced 15-30%, 24-72 h after eccentric exercise. For example, Widrick et al. (1992) had subjects cycle to exhaustion and perform eccentric knee extensions to fatigue with one leg. This eccentric exercise protocol increased muscle soreness and inflammatory cell infiltration and widened the endomysial region. Subjects then ingested 0.85 g CHO·kg body weight (bw)$^{−1}$·30 min$^{−1}$ in the 2 h after exercise and consumed a diet containing 7 g CHO·kg bw$^{−1}$·d$^{−1}$ during the 3 d after exercise. Muscle glycogen concentrations were similar between concentric and eccentric legs 6 h after exercise. However, muscle glycogen concentrations were significantly 15% and 24% lower in the eccentrically exercised leg 24 h and 72 h after exercise, respectively.

In a similar study, Costill et al. (1990) measured muscle glycogen concentrations after subjects performed concentric
cycling with both legs and 10 sets of 10 eccentric knee extensions at 120% one repetition-maximum (1RM) with one leg. This exercise protocol resulted in large increases in serum creatine kinase activity, inflammatory cell infiltration, and extreme soreness in the eccentrically exercised leg. Muscle glycogen concentrations were similar between legs 24 h after exercise. Seventy-two hours after exercise, however, muscle glycogen concentrations were significantly 32% lower in the eccentrically exercised leg. Subjects in this study (Costill et al., 1990) also consumed a diet containing either 8.5 g CHO·kg bw⁻¹·d⁻¹ or 4.3 g CHO·kg bw⁻¹·d⁻¹ during the 3 d after exercise. In both eccentric and concentric legs, muscle glycogen storage was augmented with increased carbohydrate ingestion. Muscle glycogen storage in the concentric leg, however, was always greater than in the eccentric leg, regardless of the carbohydrate content of the diet.

Other researchers have found similar results. For instance, Asp et al. (1995a) had subjects perform four 5-min bouts of one-legged eccentric cycling separated by 2 min rest periods; the other leg served as a control. One-legged eccentric cycling increased muscle soreness 48 h after exercise and caused occasional discrete signs of fiber swelling. Compared to the control leg, muscle glycogen concentrations were significantly 32% and 36% lower 24 h and
48 h after eccentric exercise, respectively.

The rate of muscle glycogen synthesis may also be reduced after eccentric exercise. Doyle et al. (1993) had subjects reduce muscle glycogen concentrations by cycling on day 1 and day 3. After cycling on day 1, subjects performed concentric or eccentric knee extensions with opposite legs. Subjects consumed 0.4 g CHO·kg bw⁻¹·15 min⁻¹ for 4 h after cycling. On day 1, the rate of muscle glycogen synthesis was similar between legs. On day 3, however, the rate of muscle glycogen synthesis was 25% lower (p<0.05) in the eccentrically exercised leg.

Reduced muscle glycogen concentrations may persist for 10 d after eccentric exercise. For example, immediately after 45 min of eccentric cycling, muscle glycogen concentrations were 39% lower than pre-exercise concentrations, but despite the consumption of a moderately high carbohydrate diet (53% of energy as carbohydrate) and complete rest, muscle glycogen concentrations remained 56% lower than baseline 10 d after exercise (O'Reilly et al., 1987). Biopsies obtained 10 d after exercise revealed severe indices of muscle damage, such as necrotic fibers and inflammatory cell infiltration, but no evidence of muscle regeneration. Biopsies, however, were obtained as close as possible to the pre-exercise biopsy incisions. It is possible that the reduced muscle glycogen
concentrations observed in this study may be the result of obtaining successive biopsies from the same area of muscle (Costill et al., 1988).

Intense exercise can also reduce muscle glycogen concentrations in well-conditioned individuals. Sherman et al. (1983) examined muscle glycogen concentrations after completion of a marathon that induced muscle soreness and damage. Muscle glycogen concentrations were reduced for 7 d after exercise despite the intake of 800 g CHO during the 24 h after exercise and the consumption of 450 g CHO·d⁻¹ for the next 6 d. Muscle glycogen concentrations were supercompensated before the marathon (196 mmol·kg wet weight⁻¹), but never rose above 60% of pre-exercise muscle glycogen concentrations (140 mmol·kg wet weight⁻¹) during the week after the marathon. Thus, even in well conditioned individuals intense exercise can produce muscle damage that is associated with reduced muscle glycogen concentrations.

It is apparent that muscle glycogen concentrations are reduced after eccentric exercise. Most studies indicate that muscle glycogen concentrations are reduced 24-72 h after eccentric exercise. Therefore, the effects of eccentric contractions on muscle glycogen concentrations should be examined 24-72 h after eccentric exercise.
Glucose Transporters and Muscle Glycogen Synthesis

Glucose uptake and transport are plasma membrane-mediated events. In vivo nuclear magnetic resonance (NMR) measurements of glycogen synthesis in man indicate that glucose transport is the rate-limiting step in glucose utilization (for review see Shulman et al., 1995). Glucose is a hydrophilic molecule that cannot cross the hydrophobic phospholipid bilayer of cell membranes. Thus, the uptake and transport of glucose into muscle tissue is regulated by a facilitated transport mechanism via a family of glucose transporters. Five functional glucose transporter isoforms (GLUT1 - GLUT5) (Klip and Paquet, 1990; Kahn, 1992) have been identified. Glucose transporters are significantly similar in structure (~65%), but most of the similarity occurs in the protein structure. The exposed antigen sites of the glucose transporters, most likely recognized by antibodies, differ significantly (Klip and Paquet, 1990).

The primary glucose transporters in mammalian skeletal muscle are GLUT1 and GLUT4 (Klip and Paquet, 1990; Kahn, 1992). GLUT1 is found in low concentrations in muscle tissue and regulates basal glucose transport (Rodnick et al., 1992). GLUT4 is found in abundant concentrations in tissues where glucose transport is markedly enhanced in response to insulin, i.e., skeletal muscle and adipose tissue (Kahn, 1992). GLUT4
is also believed to be responsible for exercise-stimulated glucose uptake (Klip and Paquet, 1990; Kahn, 1992). GLUT4 is located in subsarcolemma and intracellular sites at rest. Immunocytochemical and plasma membrane studies provide little evidence of GLUT4 transporters in the plasma membrane at rest (Rodnick et al., 1992; Borneman et al., 1992; Friedman et al., 1991). Rather, GLUT4 transporters appear to be located in or near the triads and transverse tubules, subsarcolemma vesicles and Golgi-like vesicles at rest. Most of basal GLUT4 labeling in skeletal muscle occurs in the tubulo-vesicle (T-V), trans-Golgi reticulum (TGR) or intracellular vesicles located near the sarcolemma or plasma membrane (Rodnick et al. 1992; Takata et al., 1992). Borneman et al. (1992) reported that basal GLUT4 labeling in soleus muscle is apparent in subsarcolemma vesicles, structures resembling Golgi complexes and triadic junctions. Fifty-five percent of basal GLUT4 labeling in skeletal muscle is located in the TGR complex and 2% of basal GLUT4 labeling is located in the Golgi apparatus (Blok et al., 1988). Friedman et al. (1991) observed GLUT4 labeling of human muscle in two cellular compartments: (1) within the triad on terminal cisternae and transverse tubules; and (2) within an intracellular compartment, possibly sarcoplasmic tubules (Figure 3).
Figure 3. Schematic of location of basal GLUT4.
GLUT4 is translocated to the plasma membrane after exercise or insulin stimulation. At rest, GLUT4 immunolabeling of the plasma membrane fraction of skeletal muscle is barely detectable while microsomal membranes display considerably greater immunolabeling. After insulin stimulation, however, immunolabeling of microsomal membranes is decreased while plasma membrane immunolabeling increases (Hirshman et al., 1990; Goodyear et al., 1990; Douen et al., 1989). Cell surface labeling of GLUT4 in soleus muscle also increases ~8-fold after insulin stimulation (Wilson and Cushman, 1994). Although GLUT4 labeling of the sarcolemma and plasma membrane increases after insulin stimulation, GLUT4 labeling of intracellular sites such as TGR and Golgi-apparatus are still apparent (Rodnick et al., 1992; Takata et al., 1992). Increases in the plasma membrane concentration of GLUT4 are also correlated to decreases in the intracellular concentration of GLUT4 (Douen et al., 1989; Hirshman et al., 1988; Blok et al., 1988) (Figure 4).

When plasma membrane GLUT4 is increased after insulin or muscle contraction, glucose transport and uptake increase (Hirshman et al., 1988; Douen et al., 1989, Rodnick et al., 1992; Goodyear et al. 1990; Sternlicht et al., 1989). The translocation of GLUT4 to the cell surface is closely correlated with glucose transport activity (Lund et al., 1995;
Figure 4. Schematic of location of GLUT4 after insulin- and contraction-stimulated translocation.
Kern et al., 1990; Ploug et al., 1990). Insulin stimulation and muscle contraction also have an additive effect on glucose transport. A combination of insulin stimulation and muscle contraction increases glucose transport to a greater extent than does insulin stimulation or muscle contraction alone (Wallberg-Henriksson et al., 1988; Ploug et al., 1987). It is believed that two pools of glucose transporters are present in skeletal muscle. One pool is activated by insulin stimulation and another pool is stimulated by contraction (Douen et al., 1990; Ploug et al., 1987). Labeling of GLUT4 after a combination of exercise and insulin stimulation is greater than either stimulation alone (Lund et al. 1995).

To maximize GLUT4 translocation from intracellular sites to the plasma membrane, a combination of muscle contractions and insulin stimulation must be used. An increase in plasma membrane GLUT4 increases glucose uptake. If intracellular sites of GLUT4 or plasma membrane GLUT4 concentrations are decreased after eccentric exercise, muscle glucose transport will be decreased. A decrease in glucose transport will therefore reduce muscle glycogen concentrations after eccentric exercise.

**Exercise and Delayed-Onset Muscle Soreness**

In man, performance of strenuous and unaccustomed exercise leads to delayed-onset muscle soreness (DOMS), a
condition characterized by tender, sore and damaged muscles (Schwane et al., 1983). Comparisons of concentric and eccentric muscle contractions have led to the conclusion that eccentric contractions cause the most severe DOMS and muscle damage (Asmussen, 1953; Newham et al., 1983; Schwane et al., 1983). During eccentric muscle contractions, muscle fibers lengthen while tension is developed (e.g., lowering a weight). During concentric muscle contractions, muscle fibers shorten while tension is developed (e.g., lifting a weight). Compared to concentric exercise, eccentric exercise develops more force production per fiber, recruits fewer motor units, has a lower oxygen consumption and has a reduced turnover rate of high-energy phosphates for the same absolute force production (Armstrong, 1983).

Activities such as downhill running, eccentric cycling, and eccentric muscle contractions during weight training can cause DOMS. The severity of DOMS progresses slowly over time and is usually greatest 24-72 h after a bout of unaccustomed exercise. Although a number of factors are associated with DOMS, no specific cause has been elucidated.

**Eccentric Exercise and Muscle Damage**

The intracellular storage sites of GLUT4 may be damaged after eccentric exercise. Indices of muscle damage can be observed immediately after eccentric exercise. Numerous
studies demonstrate that eccentric exercise results in an immediate and apparent disruption of muscle ultrastructure (Ogilvie et al., 1988; Hikida et al., 1983; Armstrong et al., 1986; Kuipers et al., 1983). Ultrastructural muscle damage after eccentric exercise includes muscle A-band and Z-line streaming, myofibrillar and sarcolemmal disruptions, and plasma membrane damage. Muscle A-band and Z-line lesions are apparent 30 min after downhill running in rats; muscle A-band streaming affected from two to thirty sarcomeres along the length of a fiber, and these disruptions affected the entire width of the fiber (up to 133 μm in length and 87 μm in width) (Ogilvie et al., 1988). Immediately after a marathon, Hikida et al. (1983) found evidence of ultrastructural muscle damage, such as Z-line streaming, disrupted sarcolemmas and necrotic fibers that were left with only the basal lamina outlining the former fiber.

Although ultrastructural muscle damage is apparent immediately after eccentric exercise, peak indices of muscle damage normally occur 2-3 d after eccentric exercise. For example, McCully and Faulkner (1985) found that one day after eccentric muscle contractions in mice, muscle fibers displayed signs of Z-line streaming. Two to four days later, however, macrophage infiltration and muscle fiber degeneration were apparent. Ultrastructural abnormalities were also apparent in
37% of the muscle fibers studied 2-4 d after eccentric exercise. Isometric and concentric muscle contractions in mice, however, did not demonstrate histological evidence of muscle fiber damage at any time point studied. Although widening and irregularity of cross striations and neutrophil infiltration are observed immediately after downhill running in rats, damage is most pronounced 24-48 h later (Kuipers et al., 1983). O'Reilly et al. (1987) had subjects cycle eccentrically for a total of 45 min, and 5-10% of the muscle fibers exhibited focal myofibrillar lysis, loss of Z-band material, and edema immediately after exercise. Ten days later, however, necrotic muscle fibers characterized by myofibrillar disorganization and inflammatory cell infiltration were observed. Frieden et al. (1983) had subjects cycle eccentrically, and focal disruptions of the cross-striated band pattern occurred in 32% of the fibers immediately after exercise. Three days after eccentric exercise, however, 52% of the fibers displayed focal disruptions. Focal disruptions such as muscle A-band and Z-line streaming affected more than 50% of the muscle fibers in the latter stages of damage. Frieden et al. (1981) repeatedly ran subjects down a flight of stairs, and 2 d later considerable Z-band streaming, broadening and total muscle fiber disruption were apparent. These ultrastructural
disruptions still existed 7 d after exercise.

At the present time, the mechanism(s) responsible for the reduction in muscle glycogen concentrations after eccentric exercise is(are) unknown. Eccentric exercise produces muscle damage and may decrease the amount of GLUT4 glucose transporters at rest and during exercise- and insulin-stimulated glucose uptake. Peak indices of eccentric muscle damage and reductions in muscle glycogen concentrations occur about 48-72 h after eccentric exercise. Eccentric exercise causes extensive damage to the subsarcolemma and intracellular structures of muscle fibers, including muscle A-band and Z-line streaming, mitochondrial swelling, and in severe cases, total fiber disruption and necrosis of the affected area. It is estimated that 15% or more of the fibers in a muscle may be damaged after eccentric exercise (Frieden et al., 1983). Therefore, an appreciable amount of disruption after eccentric exercise occurs in close proximity to the transverse tubules, a major storage site of GLUT4 transporters. Intracellular disruptions and Z-line streaming can also affect the entire width of a fiber, possibly damaging vesicles and Golgi-apparati that also contain GLUT4 transporters. Thus, the two primary storage sites for GLUT4 transporters may be disturbed 48-72 h after eccentric exercise. A decrease in intracellular GLUT4 would reduce the amount of GLUT4
translocated to the plasma membrane and decrease glucose transport. A decrease in insulin- or muscle contraction-stimulated glucose transport 24-72 h after eccentric exercise could reduce muscle glycogen concentrations (Figure 5).

Intracellular disruptions are not the only damage associated with eccentric exercise. There is plasma membrane damage after eccentric exercise. Plasma membrane damage might decrease the number of glucose transporters incorporated into the plasma membrane with insulin- or contraction-stimulated glucose uptake. During the 7 d after a marathon, for example, Hikida et al. (1983) observed fibers with disrupted sarcolemmas. McNeil and Khakee (1992) ran rats downhill on a treadmill and stained the fibers with a marker for rat serum albumin, a high molecular weight substance; damage to the plasma membrane permitted rat serum albumin to diffuse from the extracellular space into the cell, and compared to non-exercised control animals, rat serum albumin labeling increased nearly 7-fold 24 h after eccentric exercise. This result directly demonstrates disturbances to the plasma membrane after eccentric exercise.

Plasma membrane disruptions increase membrane permeability and typically coincide with increased plasma levels of intracellular enzymes. Increases in serum CK activity are attributed to skeletal muscle plasma membrane
Figure 5. Schematic of GLUT4 after muscle damage following eccentric exercise.
damage (Armstrong et al., 1986) and are used as an indirect marker of disruption to muscle plasma membrane integrity. Muscle CK leaks out into the blood after eccentric exercise. Serum CK activity levels can increase 20-70 times resting levels after eccentric exercise and normally peak 2-7 d after eccentric exercise (Newham et al., 1986; Evans et al., 1986). This result indirectly supports the conclusion that eccentric exercise causes plasma membrane damage.

Plasma membrane damage would also contribute to reduced muscle glycogen concentrations 24-72 h after eccentric exercise. Plasma membrane damage would decrease the number of GLUT4 transporters in the plasma membrane and decrease insulin- or muscle contraction-stimulated glucose uptake. A decrease in glucose uptake would reduce muscle glycogen concentrations 24-72 h after eccentric exercise (see Figure 2).

Evidence indicates that GLUT4 protein concentrations are decreased after eccentric exercise. Crude muscle membrane GLUT4 concentrations are reduced after eccentric exercise. Muscle glycogen concentrations and total GLUT4 protein from crude muscle membranes are decreased 24 h and 48 h after one-legged eccentric cycling in man (Asp et al., 1995a). Muscle glycogen concentrations and GLUT4 protein from crude membranes of white and red gastrocnemius muscle of rats are
also decreased 24 h and 48 h after eccentric exercise (Asp et al., 1995b). These results support the contention that decreases in GLUT4 concentrations reduce muscle glycogen concentrations after eccentric exercise.

**Muscle Glycogen Synthesis**

To optimize the rate of muscle glycogen synthesis, muscle glycogen must be depleted; GLUT4 must be translocated to the plasma membrane by exercise insulin or their combination; and blood glucose supply must not be limiting. Ingesting large amounts of carbohydrate at frequent intervals after exercise will maximize muscle glycogen synthesis. Glycogen synthesis is most rapid during the 4-6 h after exercise, and the rate of muscle glycogen synthesis is linear for at least 6 h after glycogen-depleting exercise if glucose is available (Blom et al., 1987). When large volumes (0.4 g CHO·kg bw⁻¹·15 min⁻¹) of carbohydrate are ingested after exercise, the rate of muscle glycogen synthesis may exceed 10 mmol·kg wet wt⁻¹·h⁻¹ (Doyle et al., 1993). This feeding schedule maintains high insulin concentrations that may increase plasma GLUT4 concentrations to provide adequate amounts of glucose for glycogen synthesis.

Thus, this study was undertaken to examine the role of GLUT4 transporters in reduced muscle glycogen concentrations after eccentric exercise. Muscle glycogen concentrations are reduced, total whole muscle GLUT4 protein is decreased, and
peak indices of eccentric muscle damage all occur ~48-72 h after eccentric exercise in man. Therefore, muscle glycogen concentrations, eccentric muscle damage and total GLUT4 protein content were examined 72 h after eccentric exercise.

Because eccentric contractions may damage the intracellular sites of GLUT4 transporters, basal GLUT4 transporter concentrations were measured 72 h after eccentric contractions. Eccentric muscle damage may also decrease the amount of GLUT4 transporters translocated to the plasma membrane after exercise- and insulin-stimulation. Therefore, a large volume of carbohydrate was supplied after a bout of exercise to examine exercise- and insulin-stimulated GLUT4 transporter concentration 72 h after eccentric contractions. The rate of muscle glycogen synthesis was also examined when muscle glycogen concentrations are reduced.

The purposes of this study were:

1) to determine muscle glycogen concentrations 72 h after eccentric exercise;
2) to examine muscle damage 72 h after eccentric exercise;
3) to observe basal GLUT4 transporters by immunocytochemistry 72 h after eccentric exercise;
4) to observe exercise-stimulated GLUT4 transporters by immunocytochemistry 72 h after eccentric exercise;
5) to observe insulin-stimulated GLUT4 transporters by
immunocytochemistry 72 h after eccentric exercise;

6) to determine total muscle GLUT4 protein concentration 72 h after eccentric exercise.
CHAPTER III
EXPERIMENTAL DESIGN AND METHODS

Subjects

Twenty healthy, sedentary subjects were recruited to complete the experimental protocol. Only subjects who had not engaged in regular endurance training, strength training or eccentrically-biased exercise for at least 6 months prior to the study were allowed to participate because prior exposure to eccentric exercise protects individuals from eccentric muscle damage for up to 3 months (Newham et al., 1983). All subjects were informed of the details of the experimental design, including the risks and benefits associated with participation in the study. Subjects gave their written consent in accordance with the guidelines of the Biomedical Sciences, Human Subjects Review Committee at The Ohio State University (Research Protocol #93H0112).

Preliminary Measurements

Six weeks prior to experimentation, subjects underwent preliminary testing that included a peak oxygen consumption test ($VO_{2peak}$), a one repetition maximum (1 RM) concentric knee extension test and body fat determination. A resting blood
sample was also obtained from an antecubital vein at this time.

The VO\textsubscript{2peak} test consisted of an individualized incremental exercise protocol to exhaustion on a cycle ergometer (Schwinn Biodyne, Schwinn Bicycle Company, Chicago, IL). The workload necessary to produce a heart rate of 150 bpm was initially determined. The workload was then increased by 25 W every min until exhaustion. Peak oxygen consumption was determined every 30 s during exercise using an open circuit system (REP-200B Data Acquisition System, Rayfield Equipment, Waitsfield, VT) interfaced to a Beckman LB2 CO\textsubscript{2} analyzer (Sensormedics Corporation, Anaheim, CA), an Ametek S3-A O\textsubscript{2} analyzer (Ametek, Pittsburg, PA), and a Rayfield RAM-9200 dry gas spirometer (Rayfield Equipment, Waitsfield, VT) with an Apple IIe computer. Maximal heart rates were determined via telemetry by a heart rate monitor (Polar CIC, Inc., Polar Washington, NY).

Subjects were determined to have reached VO\textsubscript{2peak} when they could no longer maintain the cycling cadence at a given workload or when they reached volitional fatigue despite strong verbal encouragement. VO\textsubscript{2peak} was defined as the mean of the two highest consecutive values of VO\textsubscript{2}. To assure that VO\textsubscript{2peak} had been reached, at least two of the following criteria had to be met: a plateau in VO\textsubscript{2} despite an increase in workload, a respiratory exchange ratio >1.10, or a heart rat
within 10 beats/min of age predicted maximal heart rate.

A concentric, single leg 1 RM knee extension was determined for each leg on a leg extension machine (Nautilus Sports/Medical Industries, Inc., Independence, VA). The concentric 1 RM was considered to be the heaviest weight the subject could lift to 150 degrees of extension. The concentric 1 RM was used to determine the workload for knee extensions during the subsequent experimental session. Height was measured on a "physicians" scale (Cardinal/Deteco Scale Manufacturing Company, Webb City, MO). Weight was measured using a digital scale (Toledo Scale, Columbus, OH). Percent body fat was calculated by a three-site skinfold measurement technique (Jackson and Pollock, 1978).

Subjects also kept detailed dietary records for a 3 d period. Because this 3 d diet was to be consumed again after the initial exercise session, subjects were instructed to consume foods they would normally eat. The 3 d dietary records were used to determine the composition and caloric content of the subjects' dietary intakes. Diets were analyzed for total energy and the percent of energy from fats, carbohydrate and proteins using a computerized dietary analysis program (Dine Systems, Inc., Buffalo, NY).

**Initial Exercise Session**

Subjects reported to the laboratory in the morning and performed concentric (CON) and eccentric (ECC) knee extensions
on a Nautilus leg extension machine using randomly assigned legs. The concentric leg (CON) performed 5 sets of 10 concentric knee extensions at 70% of concentric 1 RM. Subjects performed concentric contractions by lifting a weight with one leg; technicians lowered the weight. The CON leg served as an exercise "control" because concentric contractions induce only minimal muscle soreness and damage (Frieden et al., 1983; Newham et al., 1986). The eccentric leg (ECC) performed 10 sets of 10 eccentric knee extensions at 120% concentric 1 RM with the contralateral leg. Technicians raised the weight, and subjects performed eccentric contractions by slowly lowering the weight. This exercise protocol has been previously shown to induce muscle damage and soreness 24-72 h after exercise in the eccentrically-exercised leg (Costill et al., 1990; Widrick et al., 1992).

After knee extensions, subjects cycled for 60 min at approximately 65% VO₂peak to reduce muscle glycogen concentrations in slow-twitch muscle fibers (Ivy et al., 1988). To ensure that the subjects cycled at the correct exercise intensity, heart rates were measured periodically during cycling and maintained at approximately 70-75% of maximal heart rate. During the ride, subjects consumed water ad libitum and were cooled by fans. Five minutes after completing the 60 min cycling ride, subjects performed five 1-
min sprints at 100% VO_{peak} to reduce muscle glycogen concentrations in the fast-twitch muscle fibers (Gollnick et al., 1973).

To augment muscle glycogen synthesis after the initial exercise session, subjects consumed a carbohydrate diet containing \( \sim 6 \text{ g CHO kg}^{-1} \text{d}^{-1} \) for 3 d. The diet was based on the 3 d dietary recall. If necessary, the 3 d dietary recall was modified and a carbohydrate supplement provided (Gatorlode, Quaker Oats Company, Barrington, IL) to ensure that the subjects consumed 6 g CHO kg\(^{-1}\) d\(^{-1}\). The modified diet was eucaloric to the 3 d dietary recall. Additional energy equivalent to the energy expended during the 1 h ride was also consumed.

**Experimental Exercise Session**

Approximately 72 h hours after the initial exercise session, subjects entered the laboratory. Muscle soreness ratings for the ECC leg were reported by the subjects using by 10 point rating scale (Appendix ). A rating or "1" represented no soreness; a rating of "10" represented extreme soreness (Clarkson et al., 1986). Pre-exercise(PRE-EX) muscle biopsies were obtained (Bergstrom, 1962) from the vastus lateralis of both CON and ECC leg. Biopsies were obtained from the concentric leg first and were separated by \( \sim 5 \text{ min} \). The skin was anesthetized with \( \sim 2 \text{ ml} \) of 2% Lidocaine (HCl injection, USP: without epinephrine) and a small incision was
made through the skin and underlying fascia. Biopsies were obtained without suction. Muscle sample masses ranged from ~20-40 mg. After PRE-EX biopsies, the incisions were bandaged and pressure applied to the site for ~20 min by elastic bandages wrapped around the thigh.

Subjects then cycled for 1 h at 65% V02peak to deplete muscle glycogen. Subjects consumed water ad libitum during the ride and were cooled by fans. After cycling, the subjects rested for 45 min. The rest period minimized any transient changes in post-exercise blood glucose and insulin.

After the 45 min rest period, post-exercise (POST-EX) biopsies were obtained through the PRE-EX incisions but obtained from a different area of the muscle approximately 3 cm proximal from the first biopsy. Obtaining POST-EX biopsies in this manner avoided sampling an area of the muscle that may have been affected by the previous biopsy (Costill et al., 1988). After POST-EX biopsies, a catheter was inserted in an antecubital vein and a blood sample was drawn. The catheter was kept patent by an intravenous saline drip.

Immediately after POST-EX biopsies, subjects consumed a high carbohydrate beverage (Gatorlode, Quaker Oats Company, Barrington, IL) during the next 4 h. The carbohydrate feeding schedule provided 0.4 g CHO·kg bw\(^{-1}\) every 15 min beginning immediately after POST-EX biopsies and continuing for the next 4 h. The carbohydrate beverage contained a
mixture of maltodextrins and dextrose. Carbohydrate feedings after exercise maximize glucose transporter-translocation to the plasma membrane, stimulate insulin release, and provide ample glucose for glycogen synthesis, thereby maximizing the rate of post-exercise muscle glycogen synthesis (Doyle et al., 1993).

Blood samples were obtained every 30 min during the 4 h carbohydrate feeding period. Biopsies were obtained from CON and ECC legs at the completion of the 4 h carbohydrate feeding period (POST-CHO) and additional biopsies were obtained from a new incision 3-5 cm distal to previous biopsy incisions (Costill et al., 1988).

**Blood Analysis**

Blood samples were placed in an ice bath, allowed to clot for 30 min and centrifuged at 3000 rpm for 30 min at 4°C. Serum was removed and aliquots stored at -80°C until analysis.

Serum glucose was analyzed spectrophotometrically (Thermomax Microplate Reader, Molecular Devices, Menlo Park, CA) in duplicate using a commercially available kit (Glucose/HK, Boehringer Mannheim Diagnostics, Indianapolis, IN). Glucose-6-phosphate formed by the oxidation of glucose by hexokinase reacts in the presence of NAD⁺ and glucose-6-phosphate dehydrogenase to form NADH⁺. Glucose concentration is proportional to the absorbance of NADH⁺ measured
spectrophotometrically at 340 nm. A standard curve was developed from the absorbance and concentrations of the standards. Glucose concentrations of the unknowns were determined from this standard curve. The mean coefficient of variation for duplicate samples was 5.5%, and the inter-assay coefficient of variation was 5.1%.

Creatine kinase activity was analyzed spectrophotometrically in duplicate using a commercially available kit (Creatine Phosphokinase, Sigma Diagnostics, St. Louis, MO). Creatine kinase catalyzes the dephosphorylation of ATP in the presence of free creatine to form inorganic phosphorus. The inorganic phosphorus is then measured colorimetrically by the Fiske and SubbaRow procedure at 650 nm. A standard curve was developed from the absorbance and concentrations of the standards. Creatine kinase activities of the unknowns were determined from the standard curve. The mean coefficient of variation for duplicate samples was 4.5%, and the inter-assay coefficient of variation was 8.9%.

Serum insulin was determined in duplicate by radioimmunoassay with a commercially available kit (RSL Insulin Kit, ICN Biomedicals, Inc., Costa Mesa, CA). For each assay, a standard curve was developed from a logit-log plot by plotting insulin standards ranging from 0-200 μU/mL against the percent of radiolabelled insulin bound to the insulin antibody. The amount of radioactive ¹²⁵I in each sample was
determined using a Beckman Gamma 5500 counter with a DP5500 data reduction unit. Insulin concentrations of the unknowns were determined from the standard curve. The mean coefficient of variation for duplicate samples was 3.6%, and the inter-assay coefficient of variation was 4.2%.

**Biochemical Analysis**

**Muscle Glycogen Analysis**

The majority of each muscle sample was immediately frozen in liquid nitrogen and stored at -80°C until analysis. For analysis of muscle glycogen, a piece (~2–5 mg) of muscle was cut under liquid nitrogen and weighed to the nearest 0.01 mg (Cahn Electrobalance DTL, Cahn Instruments, Inc., Cerritos, CA). The samples were placed in 0.25 ml of 2.0 N HCl and incubated in an oven at 100°C for 2h. After reconstitution and neutralization with 0.66 N NaOH, the hydrolyzed samples were vortexed and analyzed in duplicate fluorometrically (Turner Fluorometer Model 112, Sequoia-Turner Corporation, Mountain View, CA) for glucosyl units by the method of Passoneau and Lauderdale (1974). Glycogen concentration was expressed as millimoles glucosyl units per kilogram wet muscle weight (mmol glu units⁻¹ kg wet wt⁻¹). Intra assay coefficient of variation for duplicate samples was 4.1% and inter-assay coefficient of variation was 7.6%. The mean coefficient of variation for glycogen concentration in multiple pieces of the same muscle sample was 9.5%.
Ultrastructural Muscle Analysis

Another portion of the muscle sample was prepared for ultrastructural analysis by electron microscopy. The muscle sample was placed in a solution containing 0.04% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffered saline (PBS) with sucrose (PBS/sucrose) (pH 7.4) for ~5 min. The muscle was cut into small pieces (<1 mg) and placed in a 3% glutaraldehyde/0.1 M PBS/sucrose (pH 7.4) solution for at least 2 h at 4°C. The tissue was then rinsed 3 times with PBS/sucrose, post-fixed in 1% osmium tetroxide for 1 h at 4°C and left overnight in 0.1 M PBS/sucrose. The next day the tissue was rinsed in a series of graded ethanols and propylene oxide and left in a 1:1 propylene oxide/Spurr’s resin mixture overnight. The tissue was then placed in Spurr’s resin for 3 h and embedded in latex molds overnight at 60 °C. (See Appendix G). Ultrathin longitudinal muscle sections were cut and used to determine muscle fiber ultrastructural abnormalities.

Immunocytochemistry Analysis of GLUT4

Another portion of the muscle tissue was prepared for immunocytochemistry of GLUT4. Muscle tissue was prepared as above except that the tissue was placed in degassed LR White resin for 3 h instead of Spurr’s resin. The tissue was then embedded with LR White in tin weighing pans overnight at 60 °C. (See Appendix G).
°C. Ultrathin longitudinal muscle sections were cut from LR White-embedded tissue and placed on nickel grids. The grids were initially placed tissue-side down on a drop of 5% sodium metapyrosulfate for 30 min to expose GLUT4 antigen sites. The grids were then blocked with 5% egg albumin for 2 h at room temperature. Grids were then placed tissue side down in BEEM capsules containing 50 ul of primary antiserum for 48 h at 4 °C. Primary antiserum was an affinity purified polyclonal rabbit anti-GLUT4 (East Acres Biologicals, Southbridge, MA) antibody diluted 1:200 with 0.04 M PBS containing BSA. After incubation with primary antisera, grids were placed tissue side down on a drop of 0.04 M PBS for 10 min and placed on a drop of gold labeled probe for 1 h at room temperature. The gold labeled probe was a goat anti-rabbit IgG-gold (15 nm) (AuroProbe EM GAR G15, Amersham Life Sciences, Arlington, IL) diluted 1:15 with 0.5% fish gelatin in 0.04 M PBS. Grids were washed with 0.04 M PBS, distilled water and then air dried. After drying, the grids were counterstained with uranyl magnesium acetate for 1 h and lead citrate for 2 min (See Appendix H).

Using the methods of Friedman et al. (1992), it was originally proposed that number of GLUT4 transporters in the ECC and CON legs would be quantified. However, after extensive and repeated attempts to replicate the immunocytochemical method of Friedman et al. (1992), it was
determined that this method could not be used to quantify GLUT4 in skeletal muscle in the present study (See Appendix I). Thus, a qualitative approach was undertaken.

**Muscle GLUT4 Protein Analysis**

Total muscle GLUT4 protein concentrations were determined by the method of Asp et al. (1995a). Total crude membranes were obtained by the method of Ploug et al. (1990). Approximately 10-60 mg muscle samples were homogenized twice at for 10 s in 1 ml of ice-cold buffer using a Polytron fitted with a generator (Kinematica, Switzerland). The homogenizing buffer consisted of 30 mM HEPES, 210 mM sucrose, 2 mM ethylene glycol-bis (β-aminoethylether) N,N,N′,N′-tetraacetic acid (EGTA), 40 mM NaCl and 0.35 mg/ml PMSF at pH 7.4. After homogenization, the generator was rinsed with an additional 1 ml of buffer. The homogenate was mixed with 1.5 ml of 58.3 mM sodium metaperiodate and 1.17 mM KCl and placed on ice for 15 min. The homogenate was then placed in 38.5 ml ultracentrifuge tubes, and total muscle membranes were recovered by centrifugation at 4°C for 90 min at 100,000 g in a Beckman LE-80 Ultracentrifuge with a 70.1 Ti rotor (Beckman Instruments, Palo Alto, CA). The pellet was resuspended in 400 uL of 10 mM Tris-HCl and 1 mM EDTA (pH 7.4) with a Teflon pestle (Wheaton, Millville, NJ) attached to an overhead stirrer (Wheaton Instruments, Millville, NJ). One hundred microliters of 20% SDS was added to the tube and vortexed, and
aliquots were stored at \(-80^\circ C\) for subsequent determination of protein concentration and crude muscle membrane GLUT4 concentration. (See Appendix J).

Crude muscle membrane GLUT4 concentrations were determined via electrophoresis and Western blotting. One volume of sample was mixed with one volume of solubilizing buffer and loaded onto a 10% sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gel. The solubilizing buffer contained 2% SDS, 0.002% Bromophenol blue, 0.25% glycerol, 1 mM Tris-HCL, 0.1 mM EDTA and 100 mM 1,4 dithiothreitol (DTT). Approximately 10-40 µg of protein was loaded onto each well and electrophoresed for \(-2\) h at 150 V in a horizontal Mini Gel-V 8·10 system (Gibco BRL, Gaithersburg, MD). Equivalent amounts of protein were not loaded on each well because the protein concentration of some samples was too low to allow each well to be loaded with 40 µg of protein.

After electrophoresis, proteins were transferred to Immobolin-P PVDF membranes (Millipore, Bedford, MA) by electrotransfer for 2 h at 100 V. The Immobilin-P membrane was then blocked overnight in a 5% non-fat dry milk solution at 4°C. After blocking, the membrane was incubated for 1 h at room temperature in a 1:1000 dilution of primary antibody. The primary antibody was an affinity purified polyclonal rabbit antibody produced against the last 12 COOH-terminal amino acids of the GLUT4 protein (East Acres Biologicals,
Southbridge, MA). After incubation with the primary antibody, the Immobulin-P membrane was rinsed and incubated for 1 h at room temperature with an avidin-alkaline phosphatase labeled secondary antibody solution. The membrane was then rinsed and incubated for 1 h at room temperature with a streptavidin-biotinylated alkaline phosphatase complex. Antibody-antigen complexes were visualized by immersing the membrane in a NBT/BCIP development solution (BioRad, Richmond, CA). Densitometer scanning was performed on a LKB Bromma Ultoscan XL Enhanced Laser Densitometer and Gelscan computer program (Pharmacia, Piscataway, NJ). (See Appendix K).

Individual GLUT4 protein concentrations were not measured in the present study. Because limited muscle tissue was available for GLUT4 protein analysis, muscle samples from three individuals were pooled to yield sufficient protein for GLUT4 determination. Pilot work using rat muscle indicated that at least 5 μg of protein must be loaded into each well to obtain detectable GLUT4 levels. A known amount of rat heart was also loaded onto each gel and served as an internal standard. Absorbance of the samples was expressed relative to the absorbance of the rat heart standard. The mean coefficient of variation for duplicate samples was 9.5%. GLUT4 protein concentrations were normalized as a percentage of the rat heart standard. GLUT4 protein concentrations were expressed as absorbance units per μg protein loaded.
Data and Statistical Analysis

Differences between means for muscle glycogen in the CON and ECC legs were analyzed by a two-way analysis of variance (ANOVA) with repeated measures. The independent variables were type of contraction and time of biopsy. Serum insulin, glucose and creatine kinase activity were analyzed by a one way ANOVA with repeated measures. Differences were identified by the Student-Newman-Keuls test (p<0.05).
CHAPTER IV
RESULTS

SUBJECT CHARACTERISTICS

Fourteen subjects (10 male; 4 female) completed the experimental protocol. Physical and physiological characteristics (mean ± SEM) of the subjects appear in Table 4. Individual subject characteristics are in Table 5.

Table 4. Physical and Physiological characteristics of subjects (n=14).

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>21.7 ± 0.6 y</td>
</tr>
<tr>
<td>Weight</td>
<td>71.2 ± 3.2 kg</td>
</tr>
<tr>
<td>Body Fat</td>
<td>15.4 ± 2.5%</td>
</tr>
<tr>
<td>VO_{2peak}</td>
<td>3.21 ± 0.17 L·min^{-1}</td>
</tr>
<tr>
<td></td>
<td>44.9 ± 2.0 ml·kg^{-1}·min^{-1}</td>
</tr>
<tr>
<td>Left Leg 1 RM</td>
<td>67.5 ± 4.4 kg</td>
</tr>
<tr>
<td>Right Leg 1 RM</td>
<td>67.9 ± 3.9 kg</td>
</tr>
</tbody>
</table>

(1 RM = 1 Repetition Knee Extension Maximum)
INITIAL EXERCISE SESSION

Each subject performed eccentric (ECC) knee extensions with a randomly selected leg and concentric (CON) knee extensions with the contralateral leg. Randomized CON and ECC knee extension assignments for individual subjects are shown in Appendix. Average (± SEM) weight lifted during concentric knee extensions was 46.6 ± 4.2 kg, which was ~70% of the concentric 1 RM. Average (± SEM) weight lifted during eccentric knee extensions was 82.2 ± 4.2 kg, which was ~120% of the concentric 1 RM. Weight lifted with each leg for each individual subject is shown in Table 6.

After the knee extensions, quadriceps muscle glycogen was reduced by a 1 h cycling session at 65% VO_{2peak}. The time required to complete the knee extensions plus the ride was approximately 2 h.

Diet During the 72 h After Exercise

During the 72 h after the initial exercise session, subjects consumed a moderate carbohydrate (CHO) diet and refrained from physical activity. Subjects consumed an average (± SEM) of 9.0 MJ·d^{-1} (2167 kcal·d^{-1}) and 451 ± 23 g CHO·d^{-1}. Carbohydrate consumption for each individual subject is shown in Table 7.

Rating of Muscle Soreness 72 h After Exercise

A rating of muscle soreness was obtained 72 h after the initial exercise session for each leg. The rating of muscle
soreness was determined using a standard 10 point muscle soreness rating scale (Clarkson et al., 1986) (Appendix D). The rating of muscle soreness for the ECC leg was higher than for the CON leg (p<0.05) (Figure 6). Muscle soreness ratings for each individual subject are shown in Table 8.

![RATING OF MUSCLE SORENESS](image)

**Figure 6.** Rating of muscle soreness for eccentric and concentric legs.

* = ECC leg significantly different (p<0.05) from CON.

**Muscle Damage 72 h After Exercise**

Muscle damage was determined by electron microscopy 72 h after the initial exercise session. Indices of muscle damage such as disrupted Z-lines, myofibrillar disruptions and necrotic fibers were observed 72 h after ECC exercise (Figure 7). The ECC muscle damage appeared in small, localized areas of the muscle (Figure 7). Myofibrillar
disruptions and fiber necrosis were observed in these areas of muscle damage. The CON muscle fibers did not show any evidence of muscle damage (Figure 8).

**MUSCLE BIOPSY ANALYSES**

Muscle biopsies were obtained 72 h after the initial exercise session (PRE-EX), after the second exercise session (POST-EX), and immediately after the 4 h carbohydrate feeding period (POST CHO).

**Muscle Glycogen Concentration**

Compared to CON, muscle glycogen concentrations 72 h after the initial exercise session (PRE-EX) were ~25% lower in ECC (p<0.05)(Figure 9). POST-EX and POST CHO muscle glycogen concentrations in CON and ECC were not different. Individual muscle glycogen concentrations for CON and ECC legs are shown in Table 10.

**Total Pooled GLUT4 Protein Concentration**

The total pooled muscle GLUT4 protein concentration was determined by Western blotting. An insufficient amount of muscle tissue was available for measurements of both muscle glycogen and GLUT4, so priority was given to measurements of muscle glycogen. Therefore, muscle samples from three individuals were pooled to yield sufficient protein to measure total GLUT4 protein concentration.
Figure 7. Muscle ultrastructure of eccentric leg 72 h after exercise (20,000X).
Figure 8. Muscle ultrastructure of concentric leg 72 h after exercise (20,000X)
Figure 9. Muscle glycogen concentrations 72 h after exercise, after the glycogen-depleting ride, and 4 h after the carbohydrate feeding period.

Based on the PRE-EX muscle glycogen concentration in CON and ECC, subjects were grouped into two categories as follows. The PRE-EX muscle glycogen concentration in the
CON and ECC legs was different (range = ) in six subjects, and these subjects were labeled "responders". Thus, both responders and non-responders were comprised of two groups with pooled muscle samples from three subjects. The same three subjects were also pooled at each muscle sampling time. Mean muscle glycogen concentrations 72 h after exercise in the CON of responders was 169.8 μmol·g wet wt⁻¹, whereas mean muscle glycogen concentrations in the ECC leg was 102.2 μmol·g wet wt⁻¹. The PRE-EX muscle glycogen concentration in CON and ECC was similar in six subjects, and these subjects were labeled "non-responders." Mean muscle glycogen concentrations 72 h after exercise in the CON leg of non-responders was 137.9 μmol·g wet wt⁻¹, whereas mean muscle glycogen concentration in the ECC leg were 130.7 μmol·g wet wt⁻¹.

Total pooled GLUT4 protein concentrations in CON and ECC legs were similar at all sampling times (Figure 10).

**Immunocytochemistry of GLUT4**

After repeated attempts to quantify GLUT4 (Appendix I), a qualitative approach was chosen to examine GLUT4 transporters. In longitudinal sections of muscle, GLUT4 labeling was observed near the periphery of myofibers and within the A-band (Figure 11).
Figure 10. Mean total pooled GLUT4 protein concentration of responders and non-responders 72 h after exercise, after the glycogen depleting ride, and 4 h after the carbohydrate feeding period.
Figure 11. GLUT4 labeling of longitudinal section of muscle (32,500X).
Some labeling was also observed scattered on the myofibrils within the sarcomere (Figure 11). Very little labeling was observed on the plasma membrane or t-tubules (Figure 12). In addition, mitochondria were rarely labeled (Figure 13).

In muscle cross-sections, the peripheries and plasma membranes of muscle fibers were labeled for GLUT4, with some GLUT4 labeling within the fiber (Figure 14).

There did not appear to be differences in the location of GLUT4 for CON or ECC at any sampling time.

**4 h CARBOHYDRATE FEEDING PERIOD**

Subjects consumed a carbohydrate beverage for a 4 h period after the second exercise session. The subjects ingested (± SEM) 464 ± 21 g of carbohydrate and 1739 ± 138 L of liquid during these 4 h. Subjects ingested a mean (± SEM) of 1.6 ± 0.10 g CHO•kg⁻¹h⁻¹. The absolute amount of carbohydrate consumed by each subject is shown Table 9.

**Rate of Muscle Glycogen Synthesis**

Because CON and ECC legs began the period of muscle glycogen synthesis at different muscle glycogen concentrations, an analysis of covariance (ANCOVA) was used to control for POST-EX muscle glycogen concentrations. The rates of muscle glycogen synthesis were similar in the CON and ECC legs when POST-EX muscle glycogen concentration was included as a covariant in the analysis. Muscle glycogen concentrations for subjects are shown in Table 10.
Figure 12. GLUT4 labeling of longitudinal section of the plasma membrane (32,500X).
Figure 13. GLUT4 labeling of longitudinal section of mitochondria at the plasma membrane.
Figure 14. GLUT4 labeling of cross section of muscle (32,500X).
Blood Chemistry Responses

Blood samples were drawn to examine humoral factors that are related to muscle damage and muscle glycogen synthesis. However, these results reflect the responses to both legs and are not indicative of the responses only in the ECC leg.

Creatine kinase activity was not significantly affected at any sampling time during the 4 h carbohydrate feeding period (Figure 15). One subject displayed very high creatine kinase activity 72 h after exercise, and the activity remained elevated throughout the 4 h carbohydrate feeding period; these data were excluded from analysis because the values were greater than two standard deviations from the group mean. Creatine kinase activity for each of the subjects is shown in Table 12.

The mean serum glucose concentration 30 min after beginning carbohydrate ingestion was greater than that immediate POST-EX (p<0.05) and remained elevated during the next hour of the 4 h carbohydrate feeding period (Figure 16). Blood glucose concentrations for the subjects are shown in Table 13.

The mean serum insulin concentration 90 min after beginning carbohydrate ingestion was greater than that immediately POST-EX (p<0.05) and remained elevated during the remaining 3 h of carbohydrate ingestion (Figure 16).
Insulin concentrations are shown in Table 14.

**CREATINE KINASE ACTIVITY**

![Creatine Kinase Activity Graph]

*Figure 15.* Creatine kinase activity after exercise, after the glycogen-depleting ride and every 30 min during the 4 h carbohydrate feeding period.

**SERUM GLUCOSE**

![Serum Glucose Graph]

*Figure 16.* Serum glucose concentration every 30 min during the 4 h carbohydrate feeding period.
Figure 17. Serum insulin concentration every 30 min during the 4 h carbohydrate feeding period.
The purpose of the present study was to characterize muscle glycogen concentrations and GLUT4 transporters after eccentric exercise that produces muscle damage. Muscle glycogen and total GLUT4 protein concentrations were compared in CON and ECC legs 72 h after an exercise session designed to lower muscle glycogen concentrations and produce muscle damage after eccentric exercise. In addition, GLUT4 was examined by immunocytochemistry in CON and ECC legs.

EQUAL STIMULUS FOR MUSCLE GLYCOGEN SYNTHESIS AFTER THE INITIAL EXERCISE SESSION

To compare muscle glycogen concentrations after exercise, the stimuli for muscle glycogen synthesis must be equal in both the CON and ECC legs. One factor that affects the stimulation of muscle glycogen synthesis is the exercise-induced depletion of muscle glycogen. The rate of muscle glycogen synthesis immediately after exercise is related to the exercise-induced change in muscle glycogen during exercise (Zachwieja et al., 1991).
In the present study, CON and ECC muscle glycogen concentrations were not measured immediately after the initial exercise session. However, glycogen-depleting cycling and ECC and CON knee extensions in a similar protocol resulted in equivalent post-exercise muscle glycogen concentrations for ECC and CON legs (Doyle et al., 1993). Therefore in the present investigation, there presumably was a similar exercise-induced muscle glycogen-depletion stimulus for muscle glycogen synthesis in both ECC and CON legs after the initial exercise session.

Diet and exercise were controlled during the 72 h after the initial exercise session. Subjects refrained from physical activity and consumed a diet containing a moderate amount of carbohydrate during the 72 h after the initial exercise session to augment potential differences in the muscle glycogen concentration of CON and ECC legs. Subjects consumed a mean (± SEM) of 6.3 ± 0.2 g CHO·kg BW\(^{-1}\)·d\(^{1}\) of carbohydrate.

Muscle glycogen synthesis is limited by the rate of muscle glucose uptake, which was not measured in the present study. However, the moderately high carbohydrate diet consumed after the initial exercise session should have provided sufficient carbohydrate for adequate skeletal muscle glucose uptake. Consumption of a large amount of carbohydrate
elevates blood glucose and provides a substrate for muscle glycogen synthesis. Thus, carbohydrate availability should not have limited glucose uptake during the 72 h after the initial exercise session.

Consumption of a large amount of carbohydrate produces an elevated blood insulin concentration, another important stimulus for muscle glycogen synthesis. Insulin increases muscle glucose uptake by increasing plasma membrane GLUT4 concentrations (Ploug et al., 1987; Douen et al., 1988; Blok et al., 1988; Klip and Paquet, 1990; Borneman et al., 1992). Insulin also stimulates glycogen synthase, the rate-limiting enzyme for glycogen synthesis (Doyle et al., 1993). High blood glucose and insulin concentrations induce high rates of muscle glycogen synthesis after exercise (Blow et al., 1987; Reed et al., 1989; Zachweija et al., 1991; Doyle et al., 1993). Both CON and ECC legs were exposed to similar circulating concentrations of insulin and glucose and were thus likely exposed to similar stimuli for muscle glycogen synthesis. Thus, differences in muscle glycogen concentrations 72 h after the initial exercise session were likely due to the type of contraction and the intramuscular responses to that contraction.
Numerous studies demonstrate that eccentric exercise produces muscle damage (Friden et al., 1981; Friden et al., 1983; Armstrong et al., 1983 McCully and Faulkner, 1986). Although ultrastructural muscle damage such as Z-line and A-band streaming is apparent immediately after eccentric exercise, peak indices of muscle damage occur 2-3 d after eccentric exercise. Muscle damage such as Z-line streaming, myofibrillar disruptions, a disrupted and damaged plasma membrane, and necrotic fibers are all most profound 2-3 d after eccentric exercise (Friden et al., 1983 McCully and Faulkner, 1986).

The ECC contractions were performed in the present study to produce muscle damage. The ECC leg was compared to a CON leg that had undergone presumably non-damaging concentric contractions. Thus, any muscle damage produced by ECC contractions was presumably due to the eccentric component of muscle contractions.

In the present study, fibers displaying disrupted Z-lines, disorganized myofibrillar proteins and necrotic fibers were all observed in the ECC leg 72 h after eccentric exercise (See Figure 7). Muscle damage was localized to small areas in the muscle displaying numerous indices of muscle damage (Figure 7). Intermittent and scattered areas of muscle damage
affecting only one muscle fiber or sarcomere were not observed. The CON leg showed no signs of muscle damage 72 h after the initial exercise session (Figure 8). Thus, muscle damage was produced 72 h after eccentric exercise in the present study.

Muscle damage can also be produced by improper tissue processing for electron microscopy that may produce "false" areas of muscle fiber damage. Abnormalities in mitochondrial structure and appearance in areas of muscle damage also indicate improper tissue processing. However the appearance of mitochondria in the present study was normal, even in areas of muscle damage (Figure 8). Thus, muscle damage was probably not a by-product of improper tissue processing for electron microscopy.

MUSCLE GLYCOGEN CONCENTRATIONS AFTER ECCENTRIC EXERCISE

The present study determined if, when compared to concentric exercise, muscle glycogen concentrations are reduced 72 h after eccentric exercise that produces muscle damage. One randomly assigned leg performed CON knee extensions, whereas the contralateral leg performed ECC knee extensions. Thus, the ECC leg was compared against an exercise control leg (CON) that did not produce muscle damage.

Muscle glycogen concentrations in the present study were reduced 72 h after ECC contractions that produced muscle damage when compared to a non-damaged control leg (CON)
(Figure 9). The ECC muscle glycogen concentrations were ~25% lower than CON concentrations 72 h after the initial exercise session.

Without an appropriate CON control leg, direct comparisons of muscle glycogen concentrations after exercise cannot be made. The present study, however, compared muscle glycogen concentrations in the ECC leg to a control leg that had undergone non-damaging concentric (CON) contractions 72 h earlier.

Other researchers have reported that muscle glycogen concentrations are reduced ~15-30% 24-72 h after eccentric exercise (Asp et al., 1995; Widrick et al., 1992; Costill et al, 1990). Costill et al. (1990) had subjects perform 10 sets of 10 eccentric knee extensions at 120% of 1 RM with one leg and then cycle with both legs for 60 min at 70% VO_2peak to deplete muscle glycogen. Muscle glycogen concentrations were reduced 24 h after the eccentric exercise. However, in the Costil et al. (1990) study there was an unequal stimulus for muscle glycogen synthesis after exercise because a smaller muscle glycogen depletion occurred in the eccentric leg. In a similar study, Widrick et al. (1992) had subjects cycle for 1 h at 70% VO_2peak to reduce muscle glycogen concentrations and 12 h later perform 10 sets of 10 eccentric knee extensions with one leg. Muscle glycogen concentrations were reduced 24
h and 72 h after the eccentric exercise. Also, Asp et al. (1995) had subjects cycle eccentrically with one leg, and muscle glycogen concentrations were reduced 24 h and 48 h after eccentric exercise.

**PROPOSED HYPOTHESES FOR THE REDUCED MUSCLE GLYCOGEN CONCENTRATIONS AFTER ECCENTRIC EXERCISE**

The mechanism(s) responsible for the reduction in muscle glycogen concentration during recovery from eccentric exercise versus concentric exercise is(are) unknown. A number of hypotheses have been advanced to explain the lower muscle glycogen concentrations after eccentric exercise. One hypothesis is that muscle damage produced by eccentric exercise reduces glucose uptake and this relates to muscle glycogen concentration.

Skeletal muscle membrane and sarcolemma disruptions can occur after eccentric exercise and may impair the glucose transport process. Because glucose transport is the rate-limiting step in glucose utilization and is regulated by a membrane-bound transporter, muscle membrane and sarcolemma damage may interfere with glucose transport and utilization into the cell. In the present study, muscle damage such as myofibrillar disruptions and muscle fiber necrosis were observed 72 h after ECC contractions. Any impairment in glucose transport and utilization should follow a similar
time-course as muscle damage. Peak indices of muscle damage and reduced muscle glycogen concentrations occur ~24-72 h after eccentric exercise. Thus, muscle damage may decrease glucose uptake and decrease the amount of glucose available for muscle glycogen synthesis.

It has been suggested that the membrane damage associated with eccentric exercise decreases the concentration of muscle plasma membrane GLUT4. During contraction and insulin stimulation, GLUT4 is translocated from an intracellular pool to the plasma membrane where it increases skeletal muscle glucose transport. If the plasma membrane is damaged after eccentric exercise, GLUT4 may not be translocated properly or integrated properly into the plasma membrane and the plasma membrane may be more susceptible to degradation (Asp et al., 1995). A decrease in GLUT4 concentration after eccentric exercise would decrease skeletal muscle glucose uptake, thereby reducing muscle glycogen synthesis and muscle glycogen concentration.

One investigator has reported that GLUT4 concentrations are reduced after eccentric exercise (Asp et al., 1995a; Asp et al., 1995b). Asp et al. (1995a) had subjects cycle eccentrically with one leg, and both muscle glycogen and GLUT4 protein concentrations for individual subjects were reduced 24 h and 48 h after eccentric exercise. In the present study,
total GLUT4 protein concentration was determined from pooled muscle samples of three individuals, and is possible that muscle pooling masked individual differences in GLUT4 protein concentrations between CON and ECC legs.

Total pooled GLUT4 protein concentrations were measured in the present study 72 h after the initial exercise session. Total pooled CON and ECC GLUT4 protein concentrations 72 h after the initial exercise session were not different (See Figure 10). Thus, in this study, decreases in GLUT4 after eccentric exercise did not contribute to lower muscle glycogen concentrations after eccentric exercise in the present study.

Asp et al. (1995a) has also suggested that muscle damage produced by eccentric exercise may not allow proper translocation of the GLUT4 transporter to the plasma membrane or may increase its rate of degradation. If fewer GLUT4 transporters are functional after ECC contractions, less glucose would be available for muscle glycogen synthesis. Muscle damage was apparent 72 h after eccentric exercise in the present study. Although the total pooled GLUT4 protein concentration was not different in the present study, fewer GLUT4 transporters may have been functional after ECC contractions. Muscle glucose uptake and intrinsic transporter activity, however, were not measured in the present study. Thus, it is unknown if the ECC leg contained fewer functional
GLUT4 transporters.

In addition to producing muscle damage, eccentric exercise increases the ratings of delayed-onset muscle soreness (DOMS), with the highest ratings of DOMS for muscle soreness occurring 2-7 d after exercise eccentric exercise (Clarkson et al., 1986; Schwane et al., 1983). Ratings of muscle soreness in the ECC leg were higher than in the CON leg in the present study. Using a similar protocol, Doyle et al. (1993) reported that ratings of muscle soreness 48 h after ECC contractions were higher than after CON contractions.

Blood creatine kinase activity also increases 2-7 d after eccentric exercise (Newham et al., 1986; Clarkson et al., 1986), indicating disruptions of the muscle cell membrane and/or increased cell membrane permeability. Inflammatory cells such as macrophages and neutrophils are apparent in damaged muscle 24-48 h after eccentric exercise (Armstrong et al., 1986; O'Reilley et al., 1987; Costill et al., 1990), and muscle damage produced by eccentric exercise may trigger the inflammatory response.

For example, phagocytic cells release a soluble factor that increases glycolytic flux in skeletal muscle (Shearer et al., 1988; Forster et al., 1989). Shearer et al. (1989) incubated rat skeletal muscle with harvested macrophages and reported a 22% increase in glucose uptake and an 19% increase
in the conversion of glucose to lactate. Forster et al. (1988) reported that the glycolytic flux in wounded rat skeletal muscle is increased and localized to infiltrated macrophages. A large concentration of macrophages, however, was used in both of these studies (5 X 10^6 inflammatory cells). It has been suggested that inflammatory cells may compete with muscle cells for available glucose after eccentric exercise and reduce the amount of glucose available for muscle glycogen synthesis (Costill et al., 1990; Widrick et al., 1992).

Examination of muscle tissue by electron microscopy in the present study did not reveal any inflammatory cell infiltration. Macrophages and neutrophils were not apparent in areas of muscle damage 72 h after eccentric exercise. Other researchers, however, have reported inflammatory cell infiltration after eccentric exercise. Costill et al. (1990) reported frequent sites of leukocyte infiltration 24 h after eccentric exercise. Widrick et al. (1992) observed occasional mononuclear cells engulfing what appeared to be necrotic fibers 72 h after eccentric exercise. These studies, however, examined cross-sectional sections of muscle with light microscopy after hematoxylin and eosin staining, and inflammatory cells may be more apparent using light microscopy compared to electron microscopy as in the present study.
Given that inflammatory cell infiltration was not apparent after eccentric exercise in the current study and that subjects were fed a moderately high carbohydrate diet after the initial exercise session, it is unlikely that inflammatory cells oxidized enough of the glucose entering the muscle cells to significantly reduce muscle glycogen concentrations 72 h after eccentric exercise.

Glycogen synthase is the rate-limiting enzyme for glycogen synthesis. Although glycogen synthase activity was not measured in the present study, glycogen synthase activity is not affected after eccentric exercise. Most studies report that neither the activity ratio, maximal activity nor fractional velocity of glycogen synthase are affected up to 72 h after eccentric exercise (Asp et al., 1995; Costill et al., 1990). Although not significant, Doyle et al. (1993) reported a trend for the glycogen synthase activity to be lower for eccentric exercise than concentric exercise. Thus, it is unlikely that glycogen synthase activity was impaired after ECC exercise.

**RATE OF MUSCLE GLYCOGEN SYNTHESIS 72 h AFTER EXERCISE**

The rate of muscle glycogen synthesis was also examined 72 h after eccentric exercise that produced muscle damage. This is the time when muscle glycogen concentrations are reduced after eccentric exercise. The rate of muscle glycogen
synthesis was determined during a 4 h carbohydrate feeding period after glycogen-depleting exercise.

To compare the rate of muscle glycogen synthesis in different conditions after exercise, the stimulus for muscle glycogen synthesis must be equal. Insulin is an important stimulus for muscle glycogen synthesis, because it increases skeletal muscle glucose uptake by increasing the concentration of plasma membrane GLUT4 and it stimulates glycogen synthase, the rate-limiting enzyme for glycogen synthesis. The 4 h carbohydrate feeding period increased the concentration of plasma insulin (Figure 17). Thus, both ECC and CON legs were exposed to the same circulating insulin concentration and received the same insulin-stimulation of glycogen synthesis. The 4 h carbohydrate feeding period also increased plasma glucose concentrations (Figure 16). Thus, both ECC and CON legs were exposed to the same circulating glucose concentration, i.e., similar amounts of substrate for muscle glycogen synthesis.

Collectively, these results provide strong evidence that ECC and CON legs were exposed to the same stimuli for muscle glycogen synthesis after the second session of glycogen-depleting exercise. Thus, differences in the rates of muscle glycogen synthesis must be due to the mode of contraction and intramuscular responses to the types of contractions.
The rate of muscle glycogen synthesis was 7.5 mmol glu·kg wet wt\(^{-1}\)·h\(^{-1}\) in the CON leg and 9.7 mmol glu·kg wet wt\(^{-1}\)·h\(^{-1}\) in the ECC leg (Table 11). The exercise protocol in the present study was selected to maximize any potential differences in muscle glycogen concentrations between the CON and ECC leg 72 h after the initial exercise session. Accordingly, muscle glycogen concentrations were higher in the CON leg before the start of the second exercise session. Although not significant, muscle glycogen concentrations at the completion of the second exercise session were still higher in the CON leg (Table 10).

Because the initial muscle glycogen concentration after exercise affects the rate of muscle glycogen synthesis (Zachwieja et al., 1991), an ANCOVA was used to control for the different muscle glycogen concentrations, and no effects of contraction type were detected with this analysis.

**TRANSLOCATION OF GLUT4**

The GLUT4 is located in an intracellular pool at rest and is translocated to the plasma membrane after exercise and/or insulin stimulation (Blok et al., 1988; Doen et al., 1989; Goodyear et al., 1990; Borneman et al., 1992). An increase in plasma membrane GLUT4 increases skeletal muscle glucose transport and uptake (Ploug et al., 1987; Klip and Paquet, 1990). Sternlicht et al. (1989) reported that exercise and
insulin act by different mechanisms to stimulate glucose transport in skeletal muscle. Therefore, a combination of muscle contractions and insulin stimulation is necessary to increase plasma membrane GLUT4 concentrations.

In the present study, the experimental exercise protocol provided both an exercise and an insulin stimulus for GLUT4 translocation. Subjects performed glycogen-depleting exercise and then consumed a carbohydrate beverage for 4 h after exercise. The glycogen-depleting ride provided an exercise stimulation, and the consumption of the carbohydrate beverage elevated plasma insulin (Figure 17). Both the CON and ECC leg were exposed to the same concentration of circulating insulin. Collectively, these results provide strong evidence that the CON and ECC were exposed to the same insulin and exercise stimulus for GLUT4 translocation to increase glucose uptake.

**IMMUNOCYTOCHEMISTRY OF GLUT4**

Immunocytochemistry was used to label GLUT4 for electron microscopy. In the present study, a post-embedding technique was used to label GLUT4 by immunocytochemistry. During post-embedding, tissue is initially embedded in resin; ultrathin sections of tissue are then cut and labeled with antibody for immunocytochemistry.

Friedman *et al.* (1992) used a post-embedding technique to quantify GLUT4 labeling in human skeletal muscle before and
after insulin stimulation. The GLUT4 labeling at rest was apparent on the t-tubules, terminal cisternae and on an intracellular compartment. The GLUT4 labeling at rest, however, was not observed on the plasma membrane. Insulin stimulation increased GLUT4 labeling on the t-tubules by 45% and decreased GLUT4 labeling in the intracellular compartment and on the triads by -45% (Friedman et al., 1992).

Using the post-embedding method of Friedman et al. (1992), it was originally proposed to quantify skeletal muscle GLUT4 by immunocytochemistry. However, after extensive attempts to replicate the immunocytochemical method of Friedman et al. (1992) (See Appendix I), it was determined that this method could not be used to quantify GLUT4 in human skeletal muscle. Therefore, a qualitative approach was used to assess GLUT4 in the present study.

In the present study, GLUT4 labeling was not different before or after exercise and insulin stimulation. The GLUT4 labeling in longitudinal sections of muscle occurred near the periphery of fibers and near the triads. Some scattered GLUT4 labeling was also observed in the myofibrils which is believed to reflect labelling of pieces of t-tubules (Personal communication, D. S. Whitehead, East Carolina University). The GLUT4 labeling in muscle cross-section was also occurred near the periphery of muscle, although slight labelling of
myofibrils was still apparent. The GLUT4 labeling was not present in the plasma membrane nor on mitochondria.

During post-embedding, tissue is stained with osmium tetroxide before immunocytochemistry. Osmium tetroxide is used to obtain optimal ultrastructural preservation for electron microscopy. However, osmium tetroxide reduces labeling of some antigenic proteins. Thus, osmicated tissue must be oxidized prior to immunocytochemistry to expose antigenic sites. In the present study, GLUT4 labeling was random in non-oxidized, osmicated tissue sections (Appendix). In addition, non-osmicated, post-embedded tissue almost completely loses its reactivity for GLUT4 (Borneman et al., 1992). Sodium metaperiodate is an oxidizing agent that exposes antigenic sites in osmicated tissues (Dudek and Boyne et al., 1986; personal communication, D. S. Whitehead, East Carolina University), but does not alter the surface structure of tissue (Bendayan and Zollinger, 1983).

Treatment of ultrathin sections with 5% sodium metaperiodate for 30 min exposes GLUT4 antigenic sites (Bendayan and Zollinger, 1983; personal communication with D. S. Whitehead, East Carolina University). Although stronger agents such as hydrogen peroxide and periodic acid can be used to oxidize osmicated tissue, these agents give unsatisfactory results and can etch tissue and decrease structural
preservation (Bendayan and Zollinger, 1983). Therefore, oxidation of tissue by sodium metaperiodate exposes GLUT4 antigenic sites and preserves the ultrastructure.

During post-embedding, however, only the antigenic sites exposed on the surface of the ultrathin sections are exposed to the antibody after oxidation. Therefore, the amount of antigen in the tissue must be fairly high to ensure that structures containing the antigen are labelled. Subjects performed 1 h of intense exercise, and the 4 h carbohydrate feeding period raised blood insulin concentration. Thus, both an exercise and an insulin stimulation were provided for GLUT4 translocation from intracellular sites to the cell surface. Therefore, these results suggest that GLUT4 was translocated from an intracellular storage site to the cell surface.

Most previous studies employing immunocytochemistry qualitatively assess GLUT4 transporters in skeletal muscle via a pre-embedding technique (Borneman et al., 1992; Rodnick et al., 1992; Takata et al., 1992). During pre-embedding, ultrathin sections of tissue are labeled by immunocytochemistry and then embedded in resin for electron microscopy. Unlike post-embedding, all antigenic sites are potentially exposed to the antibody.

In contrast to post-embedding, GLUT4 is translocated from intracellular sites to the plasma membrane after pre-
embedding. Borneman et al. (1992) used pre-embedding with a horse radish peroxidase-coupled antibody to examine GLUT4 in rat soleus muscle before and after insulin stimulation. At rest, heavy GLUT4 labeling was observed in subsarcolemmal vesicles, cisternae and between myofibrils. In addition, groups of labeled vesicles were also apparent between the myonucleus, on the plasma membrane and at most triadic junctions. However, after insulin stimulation nearly all of plasma membrane was heavily labeled for GLUT4. Rodnick et al. (1992) examined GLUT4 after pre-embedding in rat skeletal muscle before and after insulin stimulation. At rest, GLUT4 was observed in tubulo-vesicles, the trans-golgi reticulum and in vesicles scattered throughout the cytoplasm near the sarcolemma. No labeling was apparent on the plasma membrane. After insulin stimulation, however, there was a marked increase in labeling of the plasma membrane. Takata et al. (1992) also used pre-embedding to label GLUT4 in rat soleus muscle before and after insulin stimulation. At rest, GLUT4 labeling was present in the trans-Golgi reticulum and in vesicles located near the plasma membrane. Little labeling was observed on the plasma membrane. After insulin stimulation, however, plasma membrane and Golgi apparatus labeling increased.
Thus, numerous biochemical and immunocytochemical studies indicate that GLUT4 is translocated from an intracellular site to the plasma membrane with appropriate stimulation. Results of post-embedding immunocytochemistry in the present study do not substantiate these conclusions. GLUT4 did not appear to be translocated to the plasma membrane, nor was GLUT4 labelling altered after exercise and insulin stimulation. Thus, it appears that post-embedding was not a satisfactory method to examine GLUT4 transporters in human skeletal muscle in the present study. The GLUT4 labeling after post-embedding was markedly different than previous biochemical and pre-embedding immunocytochemistry studies.

SUMMARY

In summary, muscle glycogen concentrations were reduced 72 h after eccentric exercise that produced muscle damage in humans. Total pooled GLUT4 protein concentrations were not different between CON and ECC legs at that time. In addition, GLUT4 labeling by immunocytochemistry after post-embedding appeared to be unaltered after eccentric exercise. Therefore, alterations of skeletal muscle GLUT4 do not appear to be related to reduced muscle glycogen concentrations after eccentric exercise.
FUTURE DIRECTIONS

Future studies on this topic need to use quantitative methods to assess GLUT4 after eccentric exercise. Without quantitative measures of GLUT4, it is impossible to demonstrate that GLUT4 contributes to the reductions in muscle glycogen concentrations after eccentric exercise.

It is apparent from the results of the present study and the previous discussion that a series of follow-up studies should be performed to determine the effects of muscle damage on skeletal muscle transport and GLUT4. A pre-embedding technique needs to be developed to allow for quantitative determination of GLUT4 in skeletal muscle if muscle biopsies are to be used to assess GLUT4 after exercise.

Because the amount of muscle tissue may limit GLUT4 analyses, an animal model should be developed that produces muscle damage and reduces muscle glycogen concentrations after eccentric exercise. Sufficient muscle tissue is available in an animal model to measure glucose transporters by biochemical and immunocytochemical techniques to determine their concentrations and locations within the muscle cell to understand the regulation of glucose transport. An electrical stimulation model would allow an entire muscle or set of muscles to undergo an eccentric stimulation protocol that produces muscle damage and lowers muscle glycogen concentrations. In addition, a muscle perfusion technique
could be used. With a perfusion technique, the amount of insulin and blood glucose can be varied to maximize glucose transporter translocation and determine glucose transport.
APPENDIX A

DIAGRAM OF EXPERIMENTAL PROTOCOL
Experimental Protocol

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<th>PHASE I</th>
<th>PHASE II</th>
<th>PHASE III</th>
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<tr>
<td>0 h</td>
<td>3 d</td>
<td>1 h</td>
</tr>
<tr>
<td>ECC</td>
<td>4 h</td>
<td>4 h</td>
</tr>
</tbody>
</table>

* = Blood Draw

ECC = Eccentrically Exercised Leg (10 sets of 10 reps @ 120% 1 RM)

CON = Concentrically Exercised Leg (5 sets of 10 reps @ 70% 1 RM)

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<th>Symbol</th>
<th>Description</th>
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<tr>
<td>=</td>
<td>60 min @ 65% $\text{VO}_2\text{peak}$ with five 1 min sprints @ &gt;100% $\text{VO}_2\text{peak}$</td>
</tr>
<tr>
<td>-----</td>
<td>6 g Carbohydrate/kg/d</td>
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<tr>
<td>= 0.4</td>
<td>0.4 g Carbohydrate/kg/15 min supplement</td>
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<td>Biopsy of eccentric and concentric legs</td>
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APPENDIX B

CONSENT FORM
I, _________________________________, hereby authorize or direct W. Mike Sherman, Ph.D., Richard Strauss, M.D., David Lamb, Ph.D., or Scott Swanson, M.S. or associates or assistants of his choosing, to perform the following treatment or procedure (describe in general terms). I will ingest 800 IU/day of vitamin E or a placebo for 6 weeks prior to investigation. Determine lean body mass and percent body fat using skinfold calipers to measure the thickness of the skin. Determine the maximal rate of oxygen consumption ($V_{O_{peak}}$) and the work rate eliciting approximately 65% of $V_{O_{peak}}$ during a short, progressive intensity bicycle test to exhaustion. Undertake two exercise sessions of 1 h duration separated by 3 days. Three biopsies per leg via two incisions will be performed on the lower, outer thighs. Biopsies will be performed by Drs. Sherman or Strauss only and will be performed immediately before and after the second cycling ride and for hours after completion of the exercise. A blood sample (0.2 oz) will be obtained immediately before and after exercise and every 30 min during the feedings upon _________________________________ (myself or name of subject).

The experimental (research) portion of the treatment or procedure is: I will ingest 800 IU/day of vitamin E or a placebo for 6 weeks prior to investigation. I will be assigned to a treatment group by lottery, and neither I nor the investigator will know which supplement I am ingesting. I will perform leg extensions with a weight (concentric exercise) with one leg, while the other leg lowers a weight (eccentric exercise). I will then bicycle on a cycle ergometer for 60 min at 65% $V_{O_{peak}}$ followed by 5 one minute sprints at 100% $V_{O_{peak}}$. During the next three days I will consume a diet according to a diet exchange list that will contain a moderate amount of carbohydrate. At the end of the three day period, a muscle biopsy will be performed and I will again cycle. At the completion of the exercise, another biopsy will be performed and I will consume a carbohydrate beverage (~30 - 60 oz) Every hour for 4 hours, after which another biopsy will be performed. The muscle biopsies will be obtained in order to measure muscle glycogen concentrations as well as glucose transporters. Glucose transporters are small proteins that transport glucose into the muscle I will be monitored for a half hour after the muscle biopsy procedure to assure cessation of bleeding and lessen the risks of bruising. A total of two blood draws and placement of one catheter (a sterile, flexible teflon tube) will be performed over a four hour period, with a total of 45 ml (1.6 oz) of blood withdrawn.

This is done as part of an investigation entitled: Eccentric exercise, glucose transporters and glycogen synthesis in man.

1. Purpose of the procedure or treatment: To determine if glucose transporters are important to the replenishment of muscle glycogen in man and if vitamin E supplementation can attenuate eccentric exercise muscle damage.

2. Possible appropriate alternative procedure or treatment (not to participate in the study is always an option): I may choose not to participate and understand I am free to discontinue participation at any time during the study without any influence on my grades.

3. Discomforts and risks reasonably to be expected: Bleeding, bruising, pain and infection may occur at the site of the biopsy and blood draw.
These possibilities will be minimized by using sterile techniques and trained personnel. During the blood draw and catheter placement there is a slight possibility of light-headedness and fainting. The size of incision in the skin necessary for the biopsy procedure will be roughly 3/8 inch. Two small incisions will be made on the outer portion of each thigh. Even under normal conditions, a slight scar may develop at the site of incision of the biopsy. Thus, two small scars on each leg may develop on the outer portion of the thigh. Individuals who are predisposed to keloid formation (accumulation of scar tissue) will not be allowed to participate for cosmetic reasons. There is less than a 0.1% chance that a small portion of the biopsied muscle may lose its nerve supply and a slight chance of an adverse reaction to the local anesthetic during the biopsy procedure. During the exercise sessions the heart rate will be elevated and sweating will occur. Muscle soreness will occur after eccentric exercise, but the soreness is transient and will disappear within a few days. Consumption of the vitamin E supplement may cause gastrointestinal disturbances such as diarrhea. Consumption of the vitamin E supplement, placebo and/or diet may prove tiresome and inconvenient.

4. Possible benefits for subjects/society: The participants will learn personal physiological characteristics which are related to health and fitness including: maximal oxygen consumption, percent body fat, percentage of slow and fast twitch muscle fibers, and muscle glycogen synthesis rates. Subjects will learn how to prepare high carbohydrate meals using food exchange lists. Subjects will be paid $75 upon completion of the study. If a subject withdraws from study, the payment will be prorated.

5. Anticipated duration of subject's participation (including number of visits): Subjects receiving the vitamin E supplementation will consume the supplement or placebo for six weeks before participation. Participation in this study will last approximately 10 h of time spent in the laboratory. Subjects will report to the laboratory on 2 separate occasions. Subjects will undertake only the prescribed exercise and consume the prescribed diet.

I hereby acknowledge that _______________________ has provided information about the procedure described above, about my rights as a subject, and he/she answered all questions to my satisfaction. I understand that I may contact him/her at Phone No. 232-6887 should I have additional questions. He/She has explained the risks described above and I understand them; he/she has also offered to explain all possible risks or complications.

I understand that, where appropriate, The U.S. Food and Drug Administration may inspect records pertaining to this study. I understand further that records obtained during my participation in this study that may contain my name or other personal identifiers may be made available to the sponsor of this study. Beyond this, I understand that my participation will remain confidential.

I understand that I am free to withdraw my consent and participation in this project at any time after notifying the project director without prejudicing future care. No guarantee has been given to me concerning this treatment or procedure.
In the unlikely event of injury resulting from participation in this study, I also understand that immediate medical treatment is available at University Hospital of The Ohio State University and that the costs of such treatment will be at my expense; financial compensation beyond that required by law is not available. Questions about this should be directed to the Office of Research Risks at 292-5958.

I have read and fully understand the consent form. I sign it freely and voluntarily. A copy has been given to me.

Date:_________ Time_________ PM Signed_________________ (Subject)
Witness (es) _________________________ __________________________
If Required _________________________ (Person Authorized to Consent for Subject if Required)

I certify that I have personally completed all blanks in this form and explained them to the subject or his/her representative before requesting the subject or his/her representative to sign it.

Date:__________________________ Signed:__________________________
(Signature of Project Director or his/her Authorized Representative)
APPENDIX C

RANDOM LEG ASSIGNMENT TO ECCENTRIC AND CONCENTRIC CONTRACTIONS
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<td>R</td>
</tr>
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APPENDIX D

MUSCLE SORENESS SCALE
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APPENDIX E

DATA - INDIVIDUAL VALUES AND GROUP MEANS
TABLE 5. Physical and Physiological Characteristics of Subjects.

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<tr>
<th>Subject</th>
<th>Age (y)</th>
<th>Weight (kg)</th>
<th>Body Fat (%)</th>
<th>VO_{peak} (L min^{-1})</th>
<th>VO_{peak} (ml/kg/min)</th>
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TABLE 6. Weight Lifted During Knee Extension.

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<th>1RM-Left (kg)</th>
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<th>ECC (kg)</th>
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| Mean    | 67.85         | 67.53         | 46.6     | 82.2    |
|± SEM    | ± 3.88        | ± 4.38        | ± 4.23   | ± 4.21  |
Table 7. Carbohydrate Consumption During 3 d Diet.

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SEM 0.57 0.50
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TABLE 12. Serum Creatine Kinase Activity (nmol·min⁻¹).

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| Mean    | 51.7 | 56.4 | 43.0 | 46.0 | 45.4 | 50.0 | 41.3 | 40.9 | 41.3 | 47.7 |
| SEM     | ± 5  | ± 6  | ± 4  | ± 5  | ± 5  | ± 5  | ± 5  | ± 5  | ± 5  | ± 5  |
| ± SEM   | 29.9 | 32.3 | 28.3 | 26.0 | 25.1 | 26.8 | 25.2 | 26.5 | 28.1 | 27.7 |
Table 13. Serum Glucose (mM·L⁻¹).

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TABLE 14. Serum Insulin (µU·L⁻¹).

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Mean: 32.7 ± 10.05
SEM: 2.85 ± 12.0
Appendix F

Schematic of Rat Eccentric Exercise Device
Appendix G

Determination of Muscle Ultrastructural

by Electron Microscopy
Procedures for determination of muscle ultrastructure by electron microscopy

1. Muscle ultrastructure pre-fixative:
   
   4 mL of 0.2 M phosphate buffer with 0.2 M sucrose
   3 mL of 8% glutaraldehyde
   1 mL of ddH2O

   **DAY 1**

2. Place a drop of ultrastructure pre-fixative (may use immunocytochemistry pre-fixative) on a glass or plastic coated plate.
3. Obtain biopsy.
4. Place a piece of muscle tissue on drop of pre-fixative for "5 min. Allow tissue to harden slightly (this will "pre-fix" tissue).
5. Cut tissue with scalpel into small pieces (< 1 mg) in pre-fixative.
6. Place tissue in small jar containing 5-10 ml of EM muscle ultrastructure fixative.
7. Place tissue in 4 °C refrigerator for a minimum of 2 h.
8. After 2 h fixation period, rinse tissue 3x over a 15 min period with cold 0.1 M phosphate buffer with 0.1 M sucrose (pH 7.4). (Pipette out old buffer and pipette in new buffer).
9. Remove 0.1 M phosphate/sucrose buffer and fix tissue in 1% osmium tetroxide solution for 1 h at 4 °C.

   **1% Osmium tetroxide**
   
   1 mL of 4% Osmium tetroxide
   3 mL of 0.1 M Phosphate buffer with 0.1 sucrose at pH 7.4

   Osmium tetroxide is very damaging to the cornea. Work in fume hood!!
10. Rinse tissue 3x over a 15 min period with 0.1 M phosphate/sucrose buffer. Tissue may be stored in the refrigerator overnight in the second phosphate/sucrose buffer rinse if needed.

   **Day 2**

11. Rinse tissue 2x in graded ethanol for 5 min each rinse—50%, 70%, 80%, 95%, 100%, 100%, 100%.
12. Rinse tissue in propylene oxide for 10 min at room temperature.
13. Rinse tissue in 1:1 propylene oxide/Spurr's resin mixture
for at least 2 h. Tissue may be left in 1:1 propylene oxide/Spurr’s resin mixture overnight if needed.

**Day 3**

13. Rinse tissue 2x in Spurr’s resin for a total of 3 h.
14. Embed tissue in Spurr’s resin in latex molds overnight at 60 °C. Place a pencil written label identifying each muscle sample in each mold (embed with tissue in Spurr’s resin).
Appendix H

Determination of Muscle GLUT4

by Immunocytochemistry
Preparation of Fixative for Immunocytochemistry

(PERFORM UNDER FUME HOOD!!)

Equipment needed for immunocytochemistry fixative:

- 50 mL beaker
- Hot plate
- 1 N NaOH
- Dropper
- 8% Glutaraldehyde
- 0.2 M phosphate/sucrose buffer

NOTE:
Fixative must be made up fresh before every fixation procedure. Fixative will remain usable for that day only. Need ~3-5 mL of fixative per vial.

1. Place 2 g of formaldehyde in a beaker and add 20 mL dd H2O (Solution will be a milky white).
2. Place a stir bar in the beaker.
3. Place beaker on a hot plate set at ~ 60 °C in the fume hood (do not allow the solution to boil).
4. Heat under fume hood until paraformaldehyde vapors begin to form or bubbles form on the side of the beaker.
5. Add 1 N NaOH dropwise until paraformaldehyde solution 'turns from a milky white to clear.
6. Remove beaker from hot plate and allow solution to cool to room temperature.
7. Add the following reagents to the solution:
   - 0.625 mL of 8% glutaraldehyde solution
   - 3.75 mL of dd H2O
   - 25 mL of a 0.2 M phosphate buffer solution (pH 7.4)
   - 1.72 g of sucrose

8. Mix until dissolved and use the same day
   (makes ~50 mL of fixative)

Equipment needed for EM morphology

- 0.1 M phosphate buffer with 0.1 M sucrose at pH 7.4
- 4% osmium tetroxide
- 10 mL graduated cylinder
- Graded ethanols (50%, 70%, 80%, 95%, 100%)
- Spurr's resin
- Fume hood
- Refrigerator
Equipment needed for immunocytochemistry:

- 0.1 M phosphate buffer with 0.1 M sucrose at pH 7.4
- 4% osmium tetroxide
- 10 mL graduated cylinder
- Graded ethanols (50%, 70%, 80%, 95%, 100%)
- Medium grade LR White resin
- Aluminum weighing pans
- Transfer pipettes
- Fume hood
- Refrigerator

**Day 1**

1. Obtain muscle biopsies.
2. Place drop of immunofixative on glass or plastic coated plate.
3. Place piece of muscle biopsy in drop of immunofixative for ~5 min. Allow tissue to harden slightly (This will "pre-fix" tissue).
4. Cut tissue with scalpel into small pieces (< 1 mg) in drop of immunofixative.
5. Place tissue in small jar containing 5-10 ml of immunofixative.
6. Place tissue in 4 °C refrigerator for a minimum of 2 h.
7. After 2 h fixation period, rinse tissue 3x over a 15 min period with cold 0.1 M phosphate buffer with 0.1 M sucrose (pH 7.4). (Pipette out old buffer and pipette in new buffer).
8. Remove 0.1 M phosphate/sucrose buffer and fix tissue in 0.1% osmium tetroxide solution for 1 h at 4 °C.

**0.1% Osmium Tetroxide**

- 0.2 mL of 4% osmium tetroxide
- 7.8 mL of 0.1 M phosphate buffer with 0.1 M sucrose (pH 7.4)

Perform all procedures in the fume hood. Osmium tetroxide is very damaging to the cornea.

9. Rinse tissue 3x over a 15 min period with 0.1 M phosphate/sucrose buffer. Tissue may be stored in refrigerator overnight in the second 0.1 M phosphate/sucrose buffer rinse if needed.
Day 2

10. Rinse tissue 2x in graded ethanols for 5 min each rinse--
    50%, 70%, 80%, 95%, 100%, 100%, 100%.
11. Infiltrate tissue in de-gassed medium grade LR white resin overnight at 4°C.

Day 3

12. "Stamp Label" tin weighing pan by indentation with pen/pencil. Place tissue in tin weighing pan and embed with LR white resin. Cover tin with another aluminum weighing pan to exclude oxygen.
13. Place tin in 60 °C oven overnight.
Appendix I

Methods Attempted to Quantify Muscle GLUT4

by Immunocytochemistry
**Experiment 1:**

Muscle incubated with 1:50, 1:100, 1:200, 1:300 and 1:500 dilutions of a polyclonal primary antibody. Each dilution was then incubated with 1:15, 1:30 and 1:50 dilutions of the gold probe. Non-specific binding of the gold probe is assessed.

**Results:** Primary antibody dilution of 1:200 and 1:300 appears to give best GLUT4 labeling. Varying dilutions of gold probe did not have an effect on GLUT4 labeling. Non-specific GLUT4 labeling by the gold probe is extremely low.

**Conclusions:** Use primary antibody dilution of 1:200 with gold probe dilution of 1:15.

**Experiment 2:**

Osmicated muscle sections are incubated with 1:200, dilution of the polyclonal primary antibody and 1:15 gold probe dilution. Specific GLUT4 labeling is compared to a random grid to determine if GLUT4 labeling by the polyclonal primary antibody is specific to structures or areas in the muscle fiber.

**Results:** The majority of GLUT4 labeling occurs within the muscle fibers and is not localized to the plasma membrane, periphery of muscle fibers, or t-tubules. In addition, the distribution of GLUT4 labeling by the primary antibody is not different that of a random distribution.

**Conclusions:** GLUT4 labeling by a polyclonal primary antibody is not specific to structures or areas in osmicated sections of muscle.

**Experiment 3:**

Researchers at East Carolina who developed the GLUT4 labeling procedure are contacted. They state that osmicated tissue must be "etched" or oxidized prior to exposure to the primary antibody to expose GLUT4 antigens. In addition, they state GLUT4 labeling with a monoclonal primary antibody is more specific than a polyclonal antibody. Etching their osmicated tissue with sodium metaperiodate and using a monoclonal primary antibody has given good results.

Osmicated muscle sections are initially etched with a saturated solution of sodium metaperiodate for 60 min. Sections are the treated with a 1:200 dilution of polyclonal primary antibody and 1:15 dilution of gold probe.

**Results:** Etching the sections of muscle with a saturated solution of sodium dissolved the muscle tissue on the grids and did not produce any GLUT4 labeling.

**Conclusions:** Etching sections of muscle tissue with a saturated solution of sodium metaperiodate dissolves muscle sections from grids.
Experiment 4:
Osmicated muscle sections are etched with a 5% solution of sodium metaperiodate for 15, 30 and 60 min. Tissue is then treated with a 1:200 dilution of polyclonal primary antibody and a 1:15 dilution of gold probe.

Results: Best GLUT4 labeling occurs after 30 min of etching with a 5% sodium metaperiodate solution.

Conclusions: Etch osmicated sections of muscle tissue with a 5% solution of sodium metaperiodate for 30 min before treatment with antibody.

Experiment 5:
GLUT4 labeling is compared after polyclonal and monoclonal primary antibody application.

Results: GLUT4 labeling after polyclonal antibody application gives better results than monoclonal primary antibody application. Monoclonal antibody application increases non-specific binding.

Conclusions: GLUT4 labeling after polyclonal primary antibody application gives superior results to monoclonal primary antibody application.

Experiment 6:
Researchers at East Carolina University suggest cutting cross sections of muscle to examine GLUT4 labeling. They state GLUT4 labeling in the periphery of muscle fibers is due to labeling of sections t-tubules that are exposed after sectioning. They state that GLUT4 labeling will be localized to the periphery of muscle fibers in cross sections. Muscle cross sections are cut and labeled with the polyclonal and monoclonal antibody.

Results: GLUT4 labeling is localized near the periphery of muscle fibers, but some labeling is still apparent in the muscle fibers. In addition, less myofibrillar GLUT4 labeling is apparent after polyclonal antibody application than monoclonal antibody application.

Conclusions: GLUT4 labeling is localized near the periphery of muscle fibers in cross section, but myofibrillar labeling is still apparent. In addition, polyclonal antibody application gives superior results to monoclonal antibody application.

Experiment 7
The effects of two blocking agents are examined: egg albumin and normal goat serum. Both the polyclonal and monoclonal antibodies are applied.

Results: Egg albumin gives better blocking than normal goat serum with both polyclonal and monoclonal antibody
application.

**Conclusions:** Egg albumin should be used to block muscle sections.

**Overall Conclusions of Experiments**

Muscle sections should be blocked with egg albumin and etched with a 5% solution of sodium metaperiodate for 30 min before application of a polyclonal antibody. A 1:200 dilution of the polyclonal antibody and 1:15 dilution of the gold probe should be used for immunocytochemistry.
Appendix J

Preparation of Crude Muscle Membranes for Determination of Muscle GLUT4 Protein Concentration
(Keep homogenate on ice at all times and perform all procedures in ice cold water bath!)

1. Pre-cool rotor and ultracentrifuge before beginning procedure to 4 °C (~ 2 h in ultracentrifuge).
2. Place all homogenizations solutions on ice.
3. Grind/Pulverize dry ice and place in a container.
4. Place BAX ultracentrifuge tubes in dry ice and allow to cool. This will minimize any proteolysis that may occur.
5. Place muscle samples in dry ice.
6. Weigh frozen muscle samples on wax weighing paper with Mettler ultrabalance.
7. Record muscle weights.
8. Transfer muscle samples to pre-cooled ultracentrifuge tubes.
9. Add 1 ml of ice cold homogenate buffer to tube.

**Homogenate Buffer Solution:**
- 30 mM HEPES
- 210 mM Sucrose
- 2 mM EGTA
- 40 mM NaCl
- 0.35 mg/ml PMSF

10. Place tube in an ice/water filled beaker to minimize heat generation by polytron.
11. Polytron muscle at highest setting for 10 s. Allow homogenate to cool in ice/water bath(1 min).
12. Polytron muscle again at highest setting for 10 s.
13. Rinse polytron with 1 ml of ice cold homogenate buffer in homogenate tube(to remove residual protein from polytron and blades).
14. Place tube on ice.
15. Rinse polytron with distilled water. Run polytron through a distilled water sample to remove any excess protein from polytron. Blot dry.
16. Vortex homogenate and add 1.5 ml of ice cold KCl/Pyrophosphate to homogenate.

**KCl/Pyrophosphate Homogenate Solution**
- 1.17 M KCl
- 58.3 mM Pyrophosphate

17. Let tube stand in ice for 15 min. Vortex once during 15 min period.
18. Place tube on ultrabalance and fill tube with ice cold homogenate buffer to level of gold metal plug.
19. Record weight of tube, gold metal plug and silver plug
136

ring on balance.

21. Fill all other tubes to same weight to avoid unbalancing ultracentrifuge.

22. Tubes must be balanced and gold plugs must be properly secured. (Tubes will collapse and break in ultracentrifuge without gold metal plugs and silver ring).

23. Centrifuge at 4 °C for 90 min at 100,000 g (32,400 rpm with 70.1 Ti rotor).

24. Decant supernatant and save crude muscle membranes (stuck to inside of the tube).

25. Place tubes on ice.

26. Add 400 ul of ice cold 10 mM Tris-HCl/1 mM EDTA to ultracentrifuge tubes.

27. Place tube in ice/water bath and resuspend "pellet" by homogenization with teflon-coated pestle using tissue homogenizer set at low speed.

28. Add 100 ul of a 20% SDS solution to tube (WARNING!! Do not add SDS before resuspension. Large amounts of foam will develop during homogenization). Vortex.

29. CAUTION: SDS solution will freeze and coagulate if left on ice. SDS can be resuspended at room temperature.

30. Determine protein concentration of sample.

31. Aliquot crude muscle membrane homogenates into vials and store in -80 °C freezer until analysis.
Appendix K

Determination of Crude Muscle Membrane GLUT4 Protein Concentration by SDS-PAGE/Western Blotting
SDS-PAGE/Western blotting

Stock solutions for gels:

1. Acrylamide stock (30% acrylamide; 0.8% bis-acrylamide)
   
   1. 200 mL 30% acrylamide stock
      
      60 g acrylamide
      1.6 g bis acrylamide
      ddH2O
   
   2. Filter
   3. Place acrylamide stock in amber bottle
   4. Wrap bottle in aluminum foil
   5. Store at 4°C

   Acrylamide is a neurotoxin. Wear mask and gloves when making this solution. Always wear gloves when handling this solution!!

2. Tris buffers

   1. 500 mL of 1 M Tris, pH 8.8 at 25°C
      
      60.55 g Tris base
      -5-10 mL concentrated HCl
      ddH2O
   
   2. 500 mL of 1 M Tris, pH 6.8 at 25°C
      
      60.55 g Tris base
      -40-59 mL concentrated HCl
      ddH2O

3. 20% SDS (sodium dodecyl sulfate)

   1. 200 mL 20% SDS stock
      
      40 g SDS
      ddH2O
   
   2. Filter
   3. Store at room temperature. (SDS will precipitate in cold).

   Wear mask when making SDS. SDS can coat the lungs if inhaled.
Stock wash solutions for Western blotting:

1. **5X TBS (100mM Tris-HCl, 2.5 M NaCl, pH 7.5)**
   - Final volume 1 L
   - 292.2 g NaCl
   - 31.52 g Tris-HCl

2. **1X TBS (20mM Tris-HCl, 500mM NaCl, pH 7.5)**
   - Final volume 1 L
   - 200 mL 5X TBS
   - 800 mL ddH2O

3. **TTBS**
   - 900 mL 1X TBS
   - 450 μL Tween-20

4. **Color development buffer (100mM Tris-HCl, pH 9.5)**
   - Final volume 1 L
   - 15.76 g Tris-HCl

Pouring the gels:

1. Wash and clean all glass plates and separating bars with soap and let air dry.
2. To make 5 10% acrylamide gels (8-15 mL acrylamide/gel).
   - 22.5 mL 30% acrylamide stock (10% total)
   - 25.2 mL 1 M Tris at pH 8.8 (0.373 M total)
   - 19.46 mL ddH2O
   - 337.5 μL 20% SDS stock (0.1% total)

3. Storage buffer for gels - same ingredients without acrylamide.
   - 22.38 mL Tris at pH 8.8
   - 37.32 mL ddH2O
   - 300 μL 20% SDS

4. Construct gel pouring apparatus and casting chamber.

   - Large glass plate
   - Spacer bars
   - Smaller glass plate
   - Plastic spacer sheets
Gel casting chamber

Place glass plates in gel pouring apparatus. Place 2 plastic spacer sheets in between each glass plate. The plastic spacer sheets will make it easy to remove glass plates when gel has set-up. When 5 glass plates are in place, put in plastic displacement blocks. The top of the plastic displacement blocks should extend just over the edges of the gel casting chamber. Make sure all glass plates and spacers are aligned properly and against the bottom of the casting chamber. Place silicone gasket seal in groove in top of casting chamber. Place top of casting chamber on gel casting chamber. Tighten the wing nuts evenly. Too much pressure will break glass plates. Examine the gasket seal to make sure it is making contact and seals the entire casting chamber.

5. Make up 10% ammonium persulfate solution

0.1 g ammonium persulfate
1 mL ddH2O

Solution is viable for 1 week. Ammonium persulfate is poisonous. Wear gloves when handling.

6. When you are ready to pour the gels, add the following to the acrylamide solution:

45 µL TEMED
10% ammonium persulfate

7. Inject gel solution into gel casting chamber with a syringe. Close off the injection port. Gel should be ~6 cm high.

8. Carefully layer ddH2O on top of each gel. This will cause even polymerization.

9. Let gel polymerize for ~45-60 min (or until fully polymerized).

10. Remove gels from gel pouring apparatus and rinse gels with ddH2O.

11. Place gels in storage bag and add storage buffer. Make sure storage buffer is on top of the gels so gels do not dry out and shrink.

12. Store gels at 4° C.
Gel electrophoresis: Day 1

1. Determine amount of protein to be loaded on gel from protein concentration of the sample. 30-50 µg of protein appears to work well.

2. Pour stacking gel.

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<th>2 gels (-7.5 mL)</th>
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<tr>
<td>30% acrylamide stock</td>
<td>600 µL</td>
<td>900 µL</td>
</tr>
<tr>
<td>1 M Tris, pH 6.8</td>
<td>625 µL</td>
<td>920 µL</td>
</tr>
<tr>
<td>20% SDS stock</td>
<td>25 µL</td>
<td>37.5 µL</td>
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<tr>
<td>ddH2O</td>
<td>3.75 mL</td>
<td>5.625 mL</td>
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<tr>
<td>0.4% bromophenol blue</td>
<td>25 µL</td>
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3. Mix tracking dye

- 300 µL 20% SDS stock
- 185 µL 1 M Tris, pH 6.8
- 200 µL glycerol
- 73 µL 0.4% bromophenol blue
- 88 µL ddH2O
- 150 µL concentrated β-mercaptoethanol or (21.2 g DTT)

The tracking dye can be saved and frozen. Only useable for ~1 week before reducing agent is no longer viable.

5. Make molecular weight standard

- 1. 40 µL tracking dye
- 2. 5 µL biotinylated low MW std

Boil for 4 min in water bath and then place on ice for 15 min. Load 5-10 µl onto gel.

6. When ready to pour stacking gel, add the following to stacking gel solution.

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<td>10% Ammonium persulfate</td>
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<tr>
<td>TEMED</td>
<td>6.25 µL</td>
<td>9.4 µL</td>
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7. Pour stacking gel

Use spring clips to hold glass plates together so stacking gel will not leak. Place stacking gel in a syringe and pour stacking gel onto gel/glass plates. Carefully place well-forming comb in stacking gel. Slide comb in at an angle. Make sure there are no
bubbles trapped under comb. Leave comb in for 10-20 min or until stacking gel has polymerized. Carefully remove comb and straighten out any well walls. Wash with ddH2O.

**Gel electrophoresis:**

1. Make running buffer
   1. Final volume 1 L
   2. 3.02 g Tris base
   3. 14.42 g glycine
   4. 5 mL of 20% SDS stock

2. Place glass plates/gels in support frame. Short plates should face in the middle.
3. Gently place the gel wedge block into the support frame until it is pressed firmly against the glass plates.
4. Place electrophoresis chamber in an ice bath to keep cold.
5. Add ~750 mL of cold running buffer to electrophoresis chamber. The buffer should cover the upper electrode by ~0.5 cm.
6. Load samples (not in first or last lanes).
7. Place unit in the refrigerator.
8. Place top on unit and run at 100 V for ~15-30 min or until the tracking dye is through the stacking gel.
9. Increase the voltage to 150 V and run ~1.5 h or until the tracking dye reaches the bottom of the gel.
10. After electrophoresis, remove gels from chamber. Rinse with ddH2O.
11. Notch top corner of gel to allow for easy orientation.
12. Place gel in 50 mL of Tris-glycine transfer buffer.

**Tris-glycine transfer buffer (1.5 L)**

1. Final volume 1.5 L
2. 4.52 g Tris base
3. 21.63 g glycine
4. 300 mL methanol
5. ddH2O

Adjust pH to 8.3 with HCl at 4° C. Adjust final volume to 1.5 L

13. Equilibrate gel for 1 h in transfer buffer. This will remove salts and detergents which will shrink the gel during Western blotting.
Western Blotting

1. Cut 3 MM Whatman filter paper (2 per gel)
2. Cut Immobilin-P PVDF membranes (1 per gel)
3. Prewet filter paper and Scotch brite pads in transfer buffer.
5. Soak Immobilin-P in ddH2O for 10-15 min. Replace water at least once during the 15 min period.
7. Place blot restrainer in container with 1-2 cm of cold transfer buffer.
8. Place Scotch brite pressure pad on restrainer. Cover with filter paper.
9. Place gel on filter paper and wet with transfer buffer.
10. Place Immobilin-P on gel. Remove any bubbles between the gel and membrane. Bubbles will not allow the protein to transfer to the membrane.
12. Place pressure pad on top of filter paper.
13. Repeat these steps if another gel is to be run. (One gel appears to efficiently transfer the protein to the membrane).
14. Fill buffer tank with cold transfer buffer.
15. Place blot support frame and blot restrainer with gel in buffer tank.
16. Place blot restrainer in blot support frame, squeeze blot restrainer with gel and tighten blot clamping screw. Be sure to clamp restrainer tightly and have even pressure across membrane.
17. Place chamber in ice bath to avoid heat generation during transfer.
18. Transfer for 1.5 h at 100 V, 0.25-0.30 Amps.
19. Remove membrane from blotting apparatus and air dry.
20. Rinse the membrane 2 X 5 min at room temperature in 1X TBS.
21. Block membrane overnight at 4° C.

5 g non-fat dry milk
100 mL TTBS
Stir until dissolved
Western blotting: Day 2

1. Decant blocking solution and wash membrane for 10 min with TTBS at room temperature and gentle agitation. Decant and repeat wash 2X with additional TTBS.

2. First antibody incubation:
   Decant TTBS and add primary antibody (1:1000 dilution) solution.

   50 mL TTBS
   0.5 g non-fat dry milk
   100 µL aliquot of primary antibody
   (50 µL PA, 50 µL TTBS)

   Incubate for 1 h at room temperature with gentle agitation.

3. Decant primary antibody. Primary antibody can be used again within one week.

4. Wash membrane for 10 min with TTBS and gentle agitation. Decant and repeat wash 2X with TTBS.

5. Second antibody incubation
   Decant TTBS and add biotinylated goat anti-rabbit antibody/avidin alkaline phosphatase conjugate solution to the membrane.

   50 mL TTBS
   0.5 g non-fat dry milk
   17 µL biotinylated goat anti-rabbit antibody
   17 µL avidin alkaline phosphatase conjugate

   Incubate for 1 h at room temperature with gentle agitation.

6. Make streptavidin-biotinylated alkaline phosphatase complex. Allow complex to form for at least 1 h at room temperature (solution is not usable if complex forms for more than 3 h).

   50 mL TTBS
   17 µL streptavidin
   17 µL biotinylated alkaline phosphatase

7. Decant second antibody solution (biotinylated goat anti-rabbit/avidin alkaline phosphatase conjugate complex).

8. Add TTBS to the membrane for 10 min with gentle agitation. Decant and repeat wash 2X with TTBS.


10. Incubate for 1 h at room temperature with gentle
agitation.
11. Decant streptavidin/biotinylated alkaline phosphatase complex. The complex can be saved and analyzed for viability if no color development occurs.
12. Rinse membrane with TTBS at room temperature with gentle agitation. Decant and repeat wash 2X with TTBS.

Color development procedure:
1. Make color development solution immediately prior to use.

   25 mL color development solution
   0.25 mL NBT development solution
   0.25 mL BCIP development solution
2. Immerse membrane in color development solution with gentle agitation.
3. Time the length of color development. Protein concentrations greater than 100 ng should be visible as purple bands. Lower protein concentrations will take longer to develop.
4. Stop color development by immersing membrane in deionized water for 10 min with gentle agitation. Decant and add new water at least once during the 10 min time period (removes residual color development solution).
5. Decant and rinse membrane for 5 min with TTBS. Repeat TTBS wash (stops residual color development after membrane has been removed from solution).
6. Dry membrane between absorbent paper.
7. Purple-tint background on membrane will lighten as membrane dries.
8. Membrane should be stored in saran wrap and covered in foil to keep out light. Membrane should be read on laser densitometer as soon as possible (within three days).
9. Record results.
LIST OF REFERENCES


