IN VIVO GLUT4 TRANSLOCATION IN LEAN AND FATTY ZUCKER RAT HEARTS: EFFECT OF INSULIN DOSE AND TIME

By

Wai Ming Li

B.Sc., The University of British Columbia, 1993

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

IN THE FACULTY OF GRADUATE STUDIES

Division of Pharmacology and Toxicology
of the Faculty of Pharmaceutical Sciences

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

July 1996

© Wai Ming Li, 1996
In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Faculty of Pharmaceutical Sciences, Division of Pharmacology and Toxicology

The University of British Columbia
Vancouver, Canada

Date July 11/96
Glucose transporter type 4 (Glut4) is an isoform of facilitative glucose transporters which plays an important role in whole body glucose homeostasis. Glut4 translocation to the plasma membrane from intracellular compartments is regulated by insulin in insulin-sensitive tissues including the heart. Cardiac insulin resistance in the obese (fa/fa) Zucker rat has been characterized by depressed glucose utilization as well as glucose transport. Glucose transporter translocation has been assessed in the insulin resistant heart by measuring total glucose transporters and was shown to be responsive to insulin under *in vitro* conditions. The aim of the present study was to examine specifically the translocation of the Glut4 isoform in the heart of obese rats under *in vivo* conditions. The insulin dose effect and the time course of cardiac Glut4 translocation *in vivo* was examined in lean (FA/?) and fatty (fa/fa) Zucker rats by determining the subcellular distribution of Glut4. Plasma membrane and intracellular membrane fractions were purified from the heart ventricles isolated from rats either in the basal state or injected with insulin. Glut4 content in the membrane fractions was determined using a competitive ELISA method.

Insulin injection, given at doses 2.5, 5 and 10 U/kg, increased plasma insulin levels in both lean and fatty Zucker rats when compared to the basal level of each phenotype (p<0.05, one-way ANOVA). Insulin resistance in the fatty Zucker rat was shown by the inability of all three doses of insulin to significantly reduce plasma glucose (p>0.05, one-way ANOVA) in spite of the elevation in plasma insulin levels. On the other hand, plasma glucose was significantly reduced with all three insulin doses in lean Zucker rats (p<0.05, one-way ANOVA).

At the highest insulin dose and at 20 minutes post insulin injection, Glut4 recruitment to the plasma membrane in lean rats was shown by a significant increase (30%) in plasma
membrane Glut4 content when compared to its basal level (p<0.05, two-way ANOVA). In fatty rats, Glut4 mobilization to the plasma membrane was shown to be similarly responsive to insulin as shown by a significant and similar increase (39%) in the Glut4 content (p<0.05, two-way ANOVA). However, plasma membrane Glut4 content in the fatty Zucker rat was shown to be significantly lower than the lean phenotype in both the basal state and insulin-stimulated state (p<0.05, two-way ANOVA). In the same insulin-stimulated state, Glut4 mobilization from the intracellular membrane was shown to be responsive to insulin in the lean Zucker rat but not in the fatty Zucker rat. This was shown by a significant reduction in the Glut4 content (37%) in the lean group (p<0.05, two-way ANOVA) and no significant drop in fatty Zucker rats (p>0.05, two-way ANOVA). In the basal state, intracellular Glut4 content in fatty Zucker rats was significantly lower than lean controls (p<0.05, two-way ANOVA).

The effect of lower insulin doses on cardiac Glut4 translocation was examined and dose response curves for Glut4 mobilization to the plasma membrane and from the intracellular membrane were constructed for both lean and fatty Zucker rats. It was shown that insulin doses, at 2.5, 5, and 10 U/kg, significantly recruited Glut4 to the plasma membrane with the maximal response obtained at a dose of 2.5 U/kg for each phenotype (p<0.05, one-way ANOVA). In lean Zucker rats, Glut4 recruitment to the plasma membrane with all three insulin doses was associated with Glut4 mobilization from the intracellular store as shown by significant reductions in the Glut4 content from the basal level (p<0.05, one-way ANOVA). However in the fatty Zucker rat, Glut4 content in the intracellular membrane fraction was not significantly reduced with all three insulin doses (p>0.05, one-way ANOVA).

The time profile of cardiac Glut4 translocation showed that maximal response was already reached at 5 minutes after insulin injection and persisted for 25 minutes, as shown by no significant differences in Glut4 content among the various time points within the 25-minute period in each of the membrane fractions (p>0.05, one-way ANOVA). The time profile of
transporter translocation further substantiated the observation that insulin was able to induce Glut4 mobilization to the plasma membrane in the fatty Zucker rat without changing Glut4 content in the intracellular membrane fraction.

Evaluation of the membrane fractions using Na\(^+\)/K\(^+\) ATPase, a marker enzyme for the plasma membrane, showed high enrichment (88 fold) of the enzyme in the plasma membrane fraction when compared to the homogenate. Less than 15% contamination of plasma membrane was found in the intracellular fraction. In the fatty Zucker rat, there was a 40% reduction in the Na\(^+\)/K\(^+\) ATPase activity in the plasma membrane fraction when compared to the lean phenotype. These results may suggest an overall decrease in the enzyme activity in the insulin resistant heart, or may suggest a difference in the purity of plasma membrane fractions obtained between the lean and fatty rat.

Taken all together, the results obtained from the current study showed a lower Glut4 content in both the plasma membrane and intracellular membrane in the fatty Zucker rat heart which may suggest an overall decrease in the cardiac expression of Glut4. Cardiac Glut4 translocation in the fatty rat, as shown by recruitment of the protein to the plasma membrane, was found to be similarly responsive as the lean control. However, Glut4 mobilization to the plasma membrane in the fatty Zucker rat was shown to be not associated with a reduction in Glut4 content in the intracellular membrane fraction. This observation is not understood at present and further experiments are required to confirm this finding.
TABLE OF CONTENTS

ABSTRACT.................................................................................................................. ii
TABLE OF CONTENTS.................................................................................................. v
LIST OF TABLES ......................................................................................................... vii
LIST OF FIGURES....................................................................................................... viii
LIST OF ABBREVIATIONS........................................................................................ ix
ACKNOWLEDGMENTS............................................................................................... x
DEDICATION ................................................................................................................ xi

INTRODUCTION.......................................................................................................... 1

I) Overview of Facilitative Glucose Transporters....................................................... 1
   Isoform Distribution and Physiological Function.................................................. 3
II) Glucose Transporter Translocation......................................................................... 5
   Mechanism of Increased Glucose Transport
   in Insulin Sensitive Tissues..................................................................................... 5
   Subcellular Distribution of Glut4............................................................................ 7
   The Three Pool Model........................................................................................... 9
   Regulation of Glut4 Translocation........................................................................ 10
   Molecular Mechanism of Glucose Transporter Translocation............................ 13
III) The Genetically Obese Zucker Rat...................................................................... 16
   Insulin Resistance and Glucose Homeostasis....................................................... 16
   Cardiac Insulin Resistance in the Fatty Zucker Rat............................................. 17
   Cardiac Function of the Fatty Zucker Rat........................................................... 18
IV) Experimental Rationale and Objectives.............................................................. 19

MATERIALS AND METHODS..................................................................................... 21

I) Chemicals and Materials....................................................................................... 21
II) Animals.................................................................................................................. 22
III) Blood Sample Analyses...................................................................................... 22
IV) Subcellular Fractionation of Frozen Heart Ventricles........................................ 23
V) Ouabain-sensitive Na⁺/K⁺ ATPase Marker Enzyme Assay................................. 26
VI) Glut4 Competitive Enzyme-Linked Immunosorbant Assay (ELISA).................. 27
VII) Statistical Analysis............................................................................................ 28
RESULTS ......................................................................................................................... 30
 I) Plasma Insulin Levels After Insulin Injection ...................................................... 30
 II) Effect of Insulin Injection on Plasma Glucose Levels ..................................... 30
 III) Cardiac Glut4 Mobilization in Response to Insulin at a Single Time Point .... 41
 IV) Dose Effect of Insulin on Cardiac Glut4 Mobilization .................................. 46
 V) Temporal Effect of Insulin on Cardiac Glut4 Mobilization ............................... 51
 VI) Na+/K+ ATPase Enzyme Activity in Subcellular Fractions .............................. 56

DISCUSSION .................................................................................................................... 58
 I) Evaluation of the Subcellular Fractionation Method .......................................... 58
 II) Insulin Resistance in the Fatty Zucker Rat ......................................................... 60
 III) Lower Expression of Cardiac Glut4 in the Fatty Zucker Rat ......................... 61
 IV) Dose Effect of Insulin on Cardiac Glut4 Translocation .................................. 64
 V) Temporal Effect of Insulin on Cardiac Glut4 Translocation ............................. 65
 VI) Cardiac Glut4 Translocation Response in the Fatty Zucker Rat ..................... 67
 VII) Future Experiments ........................................................................................... 71

CONCLUSIONS ............................................................................................................... 73

REFERENCES ............................................................................................................... 74
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Tissue distribution and proposed function of facilitative glucose transporters</td>
<td>4</td>
</tr>
<tr>
<td>2.</td>
<td>Plasma insulin levels (ng/ml) of lean and fatty Zucker rats at various time points after insulin injection at a dose of 2.5 U/kg</td>
<td>31</td>
</tr>
<tr>
<td>3.</td>
<td>Plasma insulin levels (ng/ml) of lean and fatty Zucker rats at various time points after insulin injection at a dose of 5 U/kg</td>
<td>32</td>
</tr>
<tr>
<td>4.</td>
<td>Plasma insulin levels (ng/ml) of lean and fatty Zucker rats at various time points after insulin injection at a dose of 10 U/kg</td>
<td>33</td>
</tr>
<tr>
<td>5.</td>
<td>Na⁺/K⁺ ATPase activity (µmoles Pi/hr/mg) in subcellular fractions obtained from Wistar, lean and fatty Zucker rat hearts</td>
<td>57</td>
</tr>
</tbody>
</table>
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figures</th>
<th>Pages</th>
<th>Figures</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>2</td>
<td>Hypothetical model for the structure of facilitative glucose transporters.</td>
</tr>
<tr>
<td>2.</td>
<td>25</td>
<td>Subcellular fractionation of frozen heart ventricles.</td>
</tr>
<tr>
<td>3.</td>
<td>36</td>
<td>Effect of insulin injection (2.5 U/kg) on plasma glucose levels in lean and fatty Zucker rats at various time points after insulin injection.</td>
</tr>
<tr>
<td>4.</td>
<td>38</td>
<td>Effect of insulin injection (5 U/kg) on plasma glucose levels in lean and fatty Zucker rats at various time points after insulin injection.</td>
</tr>
<tr>
<td>5.</td>
<td>40</td>
<td>Effect of insulin injection (10 U/kg) on plasma glucose levels in lean and fatty Zucker rats at various time points after insulin injection.</td>
</tr>
<tr>
<td>6.</td>
<td>43</td>
<td>Plasma membrane Glut4 content in lean and fatty Zucker rat hearts in the basal state and in the insulin-stimulated state.</td>
</tr>
<tr>
<td>7.</td>
<td>45</td>
<td>Intracellular membrane Glut4 content in lean and fatty Zucker rat hearts in the basal state and in the insulin-stimulated state.</td>
</tr>
<tr>
<td>8.</td>
<td>48</td>
<td>Effect of insulin dose on plasma membrane Glut4 content in lean and fatty Zucker rat hearts.</td>
</tr>
<tr>
<td>9.</td>
<td>50</td>
<td>Effect of insulin dose on intracellular membrane Glut4 content in lean and fatty Zucker rat hearts.</td>
</tr>
<tr>
<td>10.</td>
<td>53</td>
<td>Plasma membrane Glut4 content in lean and fatty Zucker rat hearts at various time points after insulin injection.</td>
</tr>
<tr>
<td>11.</td>
<td>55</td>
<td>Intracellular membrane Glut4 content in lean and fatty Zucker rat hearts at various time points after insulin injection.</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

ATP  adenosine 5'-triphosphate
BSA  bovine serum albumin
C   centigrade
cAMP cyclic adenosine 3',5' monophosphate
EDTA  ethylene diamine tetraacetate
ELISA Enzyme-linked immunosorbant assay
GDI-2 GDP dissociation inhibitor
ip  intraperitoneal
iv  intravenous
KBS  Krebs buffered saline
kg  kilogram
l   litre
μl  microlitre
mg  milligram
min minutes
mM  millimolar
ng  nanogram
nM  nanomolar
nm  nanometer
PKC  protein kinase C
PMSF phenylmethysulfonylfluoride
PMA  phorbol myristate acetate
rpm revolutions per minute
s.e.m. standard error of the mean
TCA  trichloroacetic acid
I would like to express my thanks to Dr. John McNeill for his patience, constant encouragement, and his generosity in the course of this study. I would also like to thank my supervisory committee members, Dr. Brian Rodrigues, Dr. Roger Brownsey, Dr. Kathleen MacLeod and Dr. Ray Pederson for their constructive criticism. Special thanks to Dr. Ray Pederson for providing a portion of the animals for conducting the experiments. Special thanks also to Dr. Lyster for his skillful chairing of the committee meetings. Great appreciation to Ms. Margaret Cam for her original ideas in this project and for developing the ELISA. I would also like to thank Ms. Mary Battell for her help in finding local sources of the animals and Ms. Violet Yuen for excellent technical assistance. Great appreciation to Mr. Patrick Poucheret for constant encouragement, valuable discussion, and friendly advice. Special appreciation to my pals on the third floor and other McNeillians for constant encouragement and for making my study an unforgettable experience.
DEDICATION

I would like to dedicate this work to my loving parents who have been a guiding light throughout my life and whose love and care is my most valuable asset.
INTRODUCTION

I) Overview of Facilitative Glucose Transporters

Glucose, one of the substrates for cellular metabolism, is an energy source ubiquitous to almost all cell types including mammalian cells. Since glucose is hydrophilic, it cannot freely cross the cell membrane. The transport of glucose across the cell membrane is facilitated by transport carriers which allow the movement of glucose down its chemical gradient. In mammalian cells, the uptake of D-glucose across the plasma membrane by facilitated diffusion is mediated by a family of glucose transporters. These glucose transporters are tissue specific and play an important role in glucose homeostasis. The mammalian glucose transporter genes belong to the superfamily of transport proteins including bacterial sugar-proton symporters, bacterial transporters of carboxylic acids, and yeast sugar transporters (Mueckler, 1994). The structure of facilitative glucose transporters is illustrated in Figure 1. Common to the members of this superfamily of transport proteins are the presence of 12 α-helical transmembrane domains, a long cytoplasmic loop between the sixth and seventh transmembrane domain, an exofacial loop between the first and second transmembrane domain containing the glycosylation site, and N-terminal and C-terminal both located on the cytoplasmic side of the lipid membrane (Gould and Holman, 1993). The glucose transporter protein can be divided into two symmetrical portions by the long cytoplasmic loop between the sixth and seventh transmembrane domain. With the short loops connecting the other transmembrane domains, it is likely that six helices, rather than all twelve, are closely packed together in the lipid bilayer. Thus it is proposed that the glucose transporter protein has a bilobular structure with two groups of six transmembrane segments and a cytoplasmic tail, either the N-terminal or the C-terminal (Mueckler, 1994). In the past 15 years, a great deal of research has been done on facilitative glucose transporters to meet
the growing demand in understanding these transporters in regulating whole-body glucose homeostasis and perhaps their implications in pathological states such as diabetes.

Figure 1. Hypothetical model for the structure of facilitative glucose transporters
(Gould and Holman, 1993)
Isoform Distribution and Physiological Function

Seven glucose transporter genes (GLUT1 to GLUT7) have been identified at present (Mueckler, 1990). With different tissue distribution of the glucose transporter isoforms, different physiological roles have been assigned to each isoform (Mueckler et al., 1994). A summary of proposed functions for each of the glucose transporter isoform is illustrated in Table 1. All glucose transporter genes, except GLUT6, are expressed at the protein level (Mueckler et al., 1994). Glut1, the first to be studied, is distributed in many tissues and is responsible for transporting glucose in the basal state and during periods of starvation when plasma glucose concentration is low. Glut7 was most recently identified and is thus the least studied isoform. It has been recently shown that Glut7 is responsible for the transport of glucose produced from glucose-6-phosphate during gluconeogenesis across the endoplasmic reticulum of hepatocytes to be released into the circulation (Waddell et al., 1992). For Glut3 and Glut5 which are expressed in various tissues, there is less information available on their function and a less defined role has been proposed for them. Glut2 and Glut4 have been extensively studied for their potential role in diabetes (Mueckler, 1990; Kahn, 1992). Glut2, predominantly located in the liver and pancreatic β cells (Elliott and Craik, 1982; Orci et al., 1989), has a high capacity for glucose transport and is proposed to be responsible for glucose sensing in the pancreatic β cell. The expression of Glut2 in pancreatic β cells has been studied to elucidate the role of Glut2 in impaired β-cell insulin secretory function in diabetes (Johnson et al., 1990; Orci et al., 1990). Unlike Glut2, Glut4 is exclusively expressed in insulin-sensitive tissues including skeletal, adipose and cardiac tissues and is therefore responsible for mediating the action of insulin in its target tissues. Since Glut4 is responsible for the disposal of glucose in the postprandial state and plays a predominant role in the regulation of whole-body glucose homeostasis, it has been proposed that Glut4 may be implicated in insulin resistance in diabetes (Kahn, 1992).
Table 1. Tissue distribution and proposed function of facilitative glucose transporters

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Tissue Distribution</th>
<th>Proposed Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu1</td>
<td>many fetal and adult tissues; abundant in human red cells, endothelia, and many immortalized cell lines</td>
<td>basal glucose and increased supply for growing/dividing cells; transport glucose across blood brain barrier and other barrier tissues</td>
</tr>
<tr>
<td>Glu2</td>
<td>hepatocytes, pancreatic β cells, intestine, kidney</td>
<td>high-capacity low-affinity transport; transepithelial transport (basolateral membrane)</td>
</tr>
<tr>
<td>Glu3</td>
<td>widely distributed in human tissues; restricted to brain in other species; predominant isoform in rat placenta</td>
<td>basal transport in many human cells; uptake from cerebral fluid into brain parenchymal cells</td>
</tr>
<tr>
<td>Glu4</td>
<td>skeletal muscle, heart, adipocytes</td>
<td>rapid increase in transport in response to elevated blood insulin; important in whole-body glucose disposal</td>
</tr>
<tr>
<td>Glu5</td>
<td>intestine (jejunum), lesser amounts in adipose, muscle, brain, and kidney tissues</td>
<td>intestinal absorption of fructose and other (?) hexoses</td>
</tr>
<tr>
<td>Glu6</td>
<td>Not Expressed</td>
<td>unknown</td>
</tr>
<tr>
<td>Glu7</td>
<td>hepatocytes and other (?) gluconeogenic tissues</td>
<td>mediates flux across endoplasmic reticulum membrane</td>
</tr>
</tbody>
</table>

Adapted from Mueckler (1994)
II) Glucose Transporter Translocation

Mechanism of Increased Glucose Transport in Insulin Sensitive Tissues

As mentioned previously, Glut4 is exclusively located in insulin target tissues which include skeletal, adipose and cardiac tissues to dispose glucose in the postprandial state. The mechanism of increased glucose transport in these insulin sensitive tissues was first shown to be due to the translocation of glucose transporters to the cell-surface membrane in 1980 by Suzuki and Kono, and Cushman and Wardzala. In these studies, transporter translocation was shown by an increase and decrease in glucose transport activity in the plasma membrane and intracellular membrane respectively. The intracellular membrane fraction appeared to coincide with the fraction enriched in Golgi apparatus as indicated by the Golgi marker UDPGal:N-acetylglucosamine galactosyltransferase (Suzuki and Kono, 1980). The changes in glucose transport activity in the membrane fractions were associated with changes in glucose transporter number as determined by cytochalasin B binding studies. These studies were the first to demonstrate the translocation of glucose transporters from an unknown intracellular pool to the cell surface which could account for the increase in glucose transport due to insulin. Later studies using various insulin-sensitive tissues and various techniques, including Western blotting, immunogold labeling, and photolabeling have all confirmed that insulin increases the abundance of cell-surface Glut4 many fold by mobilizing intracellular Glut4 (Watanabe et al., 1984; Slot et al., 1991a; Slot et al., 1991b; Marette et al., 1992; Rodnick et al., 1992; Satoh et al., 1993).

In addition to the translocation of glucose transporters from an intracellular pool to the cell surface, increased intrinsic activity of glucose transporter has been proposed as one of the mechanisms for increased glucose transport caused by insulin. This was supported by two observations in biochemical studies employing subcellular fractionation techniques. First, the
magnitude of increase in cell-surface glucose transporter was found to be lower than the magnitude of increase in glucose transport (Calderhead and Lienhard, 1988; Joost et al., 1988). Therefore, the increase in glucose transporter number could not fully account for the elevation in transport activity. Hence it was proposed that perhaps there is an activation step for Glut4 to become fully functional once mobilized to the plasma membrane. Another finding which supports the existence of an activation step for glucose transporters comes from studies that showed an increase in Glut4 before the apparent increase in glucose transport in the plasma membrane. This was shown by both Western blotting (Karnieli et al., 1981) and photolabeling techniques (Gibbs et al., 1988; Yang et al., 1992) that the half time for the stimulation of Glut4 appearance on the plasma membrane was shorter than the half-time for increased glucose transport. As proposed by Holman and Cushman (1994), the difference in the timing may be due to the occlusion of newly mobilized Glut4, located near the plasma membrane, from participating in function. So far there is only one report demonstrating a change in the functional properties of cell-surface glucose transporters due to insulin. It was shown by Zaninetti et al. (1988) in the heart that insulin increased the apparent affinity of plasma membrane transporters for cytochalasin and induced positive cooperativity amongst membrane transporters as indicated by a higher Hill coefficient. However, such changes were not observed in other cytochalasin B binding studies (Cushman and Wardzala, 1980). In addition, it can be argued that the discrepancy in the magnitude of glucose transporter translocation and the increase in glucose transport may be due to cross contamination between membrane fractions obtained from subcellular fractionation. In fact, it was shown in immunocytochemical studies in cardiac and adipose tissue that insulin stimulation caused a 40 fold increase in cell-surface Glut4 (Slot et al., 1991a; Slot et al., 1991b) which can fully account for the increase in glucose transport. Therefore the suggestion that increased intrinsic activity of Glut4 is one of the mechanisms in insulin's action on glucose transport remains an area of controversy at present.
Subcellular Distribution of Glut4

The use of immunocytochemical techniques to study subcellular distribution of Glut4 was first carried out by Slot et al. (1991a; 1991b). In these studies, whole tissues were cryosectioned and Glut4 was immunolocalized using specific anti-Glut4 antibodies with gold labeling. In brown adipose tissue, Glut4 was found in several locations including tubulovesicular elements (T-V elements), endosomes, coated pits, and plasma membrane (Slot et al., 1991a). However, no Glut4 was found in the endoplasmic reticulum in adipose tissue. T-V elements are irregular, tubular membranous structures which contained the largest pool of Glut4 in the basal state. A large proportion of the Glut4-containing T-V elements were located in the Golgi complex area called trans Golgi reticulum (TGR) and were associated with stacks of Golgi cisternae. Some T-V elements were located near the plasma membrane and distributed throughout the cytoplasm. In the basal state, T-V elements contained the largest portion of Glut4 (over 80% of total Glut4) whereas the plasma membrane contained only a very small amount (~1%). The remaining portion of Glut4 was found scattered in the cytoplasm. With Glut4 redistribution in the insulin-stimulated state, ~40% of total Glut4 was found in the plasma membrane. The distribution of Glut4 in the plasma membrane appeared to be homogeneous in brown adipose tissue. The amount of Glut4 labeling on cell surface facing the endothelial cells was similar to the amount on the cell surface adjacent to the next adipose cell (Slot et al., 1991a).

In cardiac tissue, a similar subcellular distribution of Glut4 was found (Slot et al., 1991b). In the basal state, less than 1% of Glut4 was in the plasma membrane whereas over 80% was located intracellularly in T-V elements with the largest portion located in the cytoplasm and the remaining located near the sarcolemma and intercalated disk. In cardiac tissue, a smaller portion of Glut4 was located in TGR in the basal state (~13%). Also, similar to brown adipose tissue, no Glut4 was found to be located in the sarcoplasmic reticulum.
However unlike adipose tissue, cell surface Glut4 was found to be distributed unequally, with
the largest portions in the sarcolemma and T system and a lesser portion in intercalated disks
(Slot et al., 1991b). This amount was shown to be reduced by half in the insulin-stimulated
state (Slot et al., 1991b). The distribution of Glut4 in cardiac tissue was also carefully studied
by Watanabe et al. (1984) using subcellular fractionation methods. It was shown that an
insulin-sensitive intracellular fraction enriched in glucose transport activity responded to
insulin by a decrease in glucose transport activity. Detailed analysis of this membrane fraction
showed a significant difference in the sedimentation characteristics of the glucose transport
activity and of UDP-Gal:N-acetylgalactosamine galactosyltransferase activity, a marker for the
Golgi apparatus. Thus it was proposed that the Golgi apparatus may not be the membrane
structure to which intracellular glucose transport activity was associated. Hence this
intracellular membrane fraction was named the “high-speed pellet” by Watanabe et al. (1984).
Because a specific marker for the intracellular compartment has not been identified, the study
of Glut4 mobilization from the intracellular membrane has been frequently performed without
the use of any markers (Wheeler, 1988; Wheeler et al., 1994).

For skeletal muscle, the reports on the subcellular localization of Glut4 are
controversial. It has been shown in human skeletal muscle that Glut4 mobilization to the cell
surface is confined to t-tubules only, not in the sarcolemma (Friedman et al., 1991). From this
study, only one intracellular Glut4 compartment was shown. However, subsequent studies
using immunocytochemical techniques have shown Glut4 mobilization to the sarcolemma in
response to insulin (Bornemann et al., 1992; Rodnick et al., 1992). Alternatively, Glut4
mobilization to both t-tubules and the sarcolemma in response to insulin has been shown in
biochemical studies (Marette et al., 1992). For the distribution of intracellular Glut4, there is
general agreement on the location of Glut4 in T-V elements near the Golgi complex as shown
by most immunocytochemical studies (Bornemann et al., 1992; Rodnick et al., 1992; Zorzano
et al., 1996). In biochemical studies, a unique pool of insulin regulatable glucose transporter
vesicles (IRGTV) devoid of t-tubules was shown to translocate to the cell surface (Rodnick et al., 1992; Marette et al., 1992). However from immunocytochemical studies, it was shown that t-tubules in the basal state were positively and deeply stained for Glut4 suggesting that Glut4 in t-tubules may be another intracellular pool (Bornemann et al., 1992). From more recent studies, there is supporting evidence indicating the translocation of Glut4 to t-tubules by insulin (Dudek et al., 1994; Muñoz et al., 1995). Taken all together, the distribution of Glut4 in skeletal muscle is not at all clear at present. This is probably due to the presence of complex membranous structures in the skeletal muscle cell.

The Three-Pool Model

Since the first demonstration of glucose transporter translocation from an intracellular compartment to the plasma membrane by Suzuki and Kono, and by Cushman and Wardzala in 1980, a simple two-pool model had been proposed. Recently a new method for the detection of Glut4 has been developed which can allow the estimation of transporter translocation in intact cells. Using bis-mannose photolabel ATB-BMPA (Holman et al., 1990) and 1,3-bis-(3-deoxy-D-glucopyranose-3-yloxy)-2-propyl 4-benzoylbenzoate (B3GL) (Jhun et al., 1992) as photolabels, glucose transporter subcellular trafficking could be studied by following the movement of photolabeled Glut4 in fully-stimulated cells. This made possible the study of glucose transporter internalization and redistribution in the steady-state (Clark et al., 1991; Jhun et al., 1992; Satoh et al., 1993). In both the basal and insulin-stimulated state, it was found that Glut4 was continuously recycled from the cell surface to the inside of the cell without a net movement of Glut4 between the intracellular compartment(s) and the cell surface (Jhun et al., 1992). It was also proposed that the abundance of cell surface Glut4 was determined by the balance of endocytosis and exocytosis and that the action of insulin is by promoting exocytosis and/or inhibiting endocytosis (Jhun et al., 1992; Satoh et al., 1993; Yang et al., 1993). In addition, the rate constants for endocytosis and exocytosis determined
from these studies allowed the modeling of the Glut4 trafficking process using computer simulation (Holman et al., 1994). It was shown that a simple two-pool model could not fully account for the observation of the slow steady-state recycling of Glut4 during insulin stimulation while the initial appearance of Glut4 on the cell surface was rapid from the basal to insulin-stimulated state. Hence, it was proposed that Glut4 is recycled via the endosomal recycling pathway in which Glut4 is internalized into early endosomes and then consecutively sorted back into the storage pool before reappearing on the cell surface. The sorting of endocytosed Glut4 into the storage pool is further supported by the findings from immunocytochemical studies (Slot et al., 1991a; Slot et al., 1991b). In the insulin-stimulated state, endosomes stained positive for Glut4 were also stained positive for albumin, an indicator of early endosomes. It was observed that some endosomes were connected with elongated tubular structures of T-V elements. In these endosomal-tubulo structures, albumin was restricted to the endosome part whereas Glut4 was enriched in the tubular part (Slot et al., 1991a). These observations suggest that Glut4 is recycled by endocytosis and then sorted back into T-V elements, the largest intracellular pool. Very recently, kinetic studies using chimeric forms of Glut1 and Glut4 also supported a 3-pool model based on the rates of internalization and the subcellular distribution of transporters (Verhey et al., 1995). Taken all together, it is proposed that there are at least two intracellular pools of glucose transporter.

Regulation of Glut4 Translocation

The most well known stimulus of Glut4 translocation is insulin since its first demonstration in adipocytes by Suzuki and Kono, and Cushman and Wardzala in 1980. In addition to the demonstration in vitro and in vivo using pharmacological concentrations of insulin, transporter translocation in response to insulin physiologically has been shown in skeletal muscles using an oral glucose load as the primary stimulus in vivo (Napoli et al., 1995). In addition to insulin, glucose itself has been shown to induce its own transport by
mobilizing glucose transporters to the cell-surface membrane (Zaninetti et al., 1988; Galante et al., 1995). It has been shown in heart perfusion experiments that 3-O-methyl glucose, at a concentration of 15 mM, enhanced glucose transport and transporter translocation as measured by cytochalasin B binding (Zaninetti et al., 1988). In addition to cardiac tissue, it has been shown in skeletal muscle that acute hyperglycemia could induce Glut4 translocation both under in vitro and in vivo conditions with the effect comparable to that of insulin (Galante et al., 1995). The mechanism of glucose-induced translocation is not well understood at present. However, preliminary evidence suggests that the activation of protein kinase C (PKC) may be involved (Galante et al., 1995). Palmitate, another energy source, has been shown to have differential roles in glucose transport in different tissues (Hardy et al., 1991). In isolated adipocytes, glucose transport was responsive to palmitate with the movement of intracellular Glut4 to the plasma membrane (Hardy et al., 1991). In skeletal muscle (Hardy et al., 1991) and cardiac muscle (Wheeler et al., 1994), on the other hand, palmitate was shown to have inhibitory effects on glucose transport. The mechanism of the inhibition may be due to altered distribution of Glut4 caused by palmitate (Wheeler et al., 1994).

Second messengers also play a role in the regulation of Glut4 translocation. Glucose transporter translocation has been shown to be regulated by phorbol esters, stimulators of PKC (Vogt et al., 1990; Saltis et al., 1991; Vogt et al., 1991; Nishimura et al., 1994). In these studies, both 3-O-methyl glucose transport and Glut4 translocation was induced by phorbol esters. However, the effect was found to be less than that of insulin. At present, the role of PKC in glucose transporter translocation and its mechanism remains unclear. Staurosporine, a PKC inhibitor, was able to inhibit both insulin-stimulated and phorbol ester-stimulated glucose transport and Glut4 translocation (Nishimura et al., 1994) suggesting a possible role of PKC in insulin signaling to glucose transporter translocation. In addition to PKC, cyclic AMP has been shown to regulate glucose transporter translocation (Kelada et al.,
Similar to PKC, cAMP can enhance glucose transport, as measured by 2-deoxyglucose transport, by causing the redistribution of Glut4 to the cell-surface. However, at high concentrations of cAMP, the action of cAMP is complex. It was shown that Glut4 translocation was stimulated while glucose transport was reduced by 1mM dibutyryl cAMP (Macaulay et al., 1994). Hence the role of cAMP in the glucose transport system appears to be far more complicated and its mechanism of action remains to be elucidated.

Metabolic state and hormonal state are also regulators of glucose transporter translocation as shown by in vitro experiments. Under hypoxic or anoxic conditions, Glut4 mobilization from the intracellular store to the sarcolemma was shown in perfused heart experiments (Wheeler, 1988; Sun et al., 1994). Increased workload has also been shown to induce Glut4 translocation in perfused hearts (Zaninetti et al., 1988; Wheeler et al., 1994). Furthermore, as shown in cardiac myocytes and skeletal muscles, contractile activity also had positive effects on Glut4 translocation (Kolter et al., 1992; Gao et al., 1994; Etgen et al., 1993). Other conditions, such as high pH and hyperosmolarity, have been shown to cause an apparent translocation of glucose transport activity in isolated adipocytes (Toyoda et al., 1986). In addition, Glut4 translocation has been shown to be induced by IGF-I (Wilson et al., 1995) indicating the hormonal regulation of Glut4 translocation is not restricted to insulin. As well, in vitro experiments have shown that Glut4 translocation was responsive to catecholamines in perfused heart (Rattigan et al., 1991). Since the effects of catecholamines on metabolism are opposite to insulin, the physiological significance of this finding needs to be substantiated by further experiments.
Molecular Mechanism of Glucose Transporter Translocation

The molecular mechanism of glucose transporter translocation is a current research area of interest. The different trafficking behavior of the two isoforms Glut1 and Glut4 present in insulin-sensitive tissues suggests that the amino acid sequence of the transporter protein may play a role in its targeting as initially shown by Haney et al. (1991) and Hudson et al. (1992). To identify possible targeting regions, chimeric forms of Glut1 and Glut4 have been expressed in several cell types to study their subcellular distribution. It was shown by Piper et al. (1992) that the NH2 terminus of Glut4 conferred intracellular sequestration. Further, it has been shown that a dileucine motif in the COOH terminus was a signal for Glut4 endocytosis and for intracellular sequestration (Corvera et al., 1994; Verhey and Birnbaum, 1994). The importance of the COOH terminus for the determination of cellular localization of Glut4 was further supported by Czech et al. (1993), Verhey et al. (1993; 1995), and Haney et al. (1995). In addition, Smith et al. (1991) have shown that the unmasking of the COOH terminus of Glut4 during translocation may be part of the mechanism of insulin-stimulated glucose transport in rat adipocytes, again supporting the importance of the COOH terminus. On the contrary, Asano et al (1992) have provided evidence to indicate that neither the NH2 nor the COOH terminus contained regions for intracellular targeting. The controversial results obtained from these studies were probably due to experimental limitations as discussed by Holman and Cushman (1994). For example, most of the cell types used were not insulin sensitive and a very high level of expression was produced from the transfection techniques. Thus the accumulation of chimeric transporters may not reflect the distribution of the native forms of the transporter in insulin-sensitive cells. In addition, the difference in the distribution and targeting of Glut1 and Glut4 is quantitative while most transfection studies could only provide qualitative results on the distribution of the chimeric forms of glucose transporter.
The elucidation of the targeting region of the glucose transporter may provide information on the differential distribution of transporters regulated by insulin. However, it does not explain the molecular mechanism of the translocation process. Thus current experiments are aimed at finding molecules in Glut4 vesicles which may provide clues about the machinery of the translocation process. Current experimental findings suggest that the movement of Glut4 vesicles may be similar to the regulated secretory pathway. Small molecular weight GTP-binding proteins (20-30 kDa), which are known to be involved in the regulation of exocytotic and endocytotic pathways (Balch, 1990) have been shown to be present in Glut4-containing vesicles in adipocytes (Cormont et al., 1991; Cormont et al., 1993) and in cardiac muscle (Uphues et al., 1994). The role of small G-proteins in Glut4 vesicle trafficking was substantiated by the findings that a nonhydrolyzable analog of GTP, GTPγS, could induce Glut4 translocation in permeabilized 3T3-L1 adipocytes (Robinson et al., 1992) and rat adipocytes (Baldini et al., 1991). The translocation of Glut4 in skeletal muscle was found to be associated with the translocation of small G-proteins to the plasma membrane from intracellular vesicles enriched in Glut4 (Etgen et al., 1993), further supporting the role of small G-proteins in the transport of Glut4 vesicles. Recently, the small G-protein involved in Glut4 vesicle trafficking has been identified to be Rab4 in adipocytes (Cormont et al., 1993) and Rab4A in cardiac muscle (Uphues et al., 1994). Shisheva et al. (1994a) has recently shown the involvement of an isoform of GDP dissociation inhibitor (GDI-2) in the action of insulin on membrane movements. It was shown that GDI-2 was colocalized with Glut4 in low density microsomes of 3T3-L1 adipocytes. In addition, Glut4 and GDI-2 content in low density microsomes was decreased in response to insulin stimulation. This was associated with an increase in Glut4 content in the plasma membrane while GDI-2 content remained unchanged, suggesting the release of GDI-2 into the cytoplasm in response to insulin (Shisheva et al., 1994a). Since GDI-2 has been shown to interact with Rab4 it was suggested that GDI-2 may be involved in the regulation of Glut4 vesicle trafficking (Shisheva et al., 1994b).
In addition to small G-proteins, the discovery of other trafficking proteins colocalizing in Glut4 vesicles suggests that Glut4 vesicle trafficking is similar to that of regulated secretion such as neurotransmitter release at synapses and hormone release from endocrine cells. Cain et al. (1992) and Laurie et al. (1993) have shown the presence of synaptobrevin, a protein involved in vesicle trafficking for synaptic transmission, in Glut4-containing vesicles in adipocytes. It was also shown that synaptobrevin was translocated to the plasma membrane in response to insulin (Cain et al., 1992; Laurie et al., 1993). In the current hypothesis of synaptic vesicle fusion process, synaptobrevin is part of the fusion apparatus involved in the docking of vesicles to the cell-surface membrane (Söllner et al., 1993; Takizawa and Malhotra, 1993). Hence it might be possible that the fusion of Glut4 vesicle with the plasma membrane is brought about by synaptobrevin which serves as a docking protein for Glut4 vesicles. The recycling pathway of Glut4 may also resemble the conventional endocytotic pathway as two proteins typically found in recycling endosomes, secretory carrier membrane proteins (SCAMPs) (Laurie et al., 1993; Thoidis et al., 1993) and cellubrevin (Volchuk et al., 1995) have been shown to localize in Glut4 vesicles.

In addition to the discovery of small G-proteins and possible docking proteins which suggest the translocation process may be similar to regulated secretion, various other proteins have been shown to localize in Glut4 vesicles and may be involved in the translocation process of Glut4. These proteins include a 165-kDa protein (Mastick et al., 1994), a glycoprotein of Mr 160,000 (Kandror and Pilch, 1994), a 70-kDa cytosolic protein (GTBP70) (Liu et al., 1995), and caveolin (Scherer et al., 1994; Kandror et al., 1995). The role of these various proteins in Glut4 vesicle trafficking is presently unknown and needs to be substantiated by future experiments.
III) The Genetically Obese Zucker Rat

The fatty Zucker rat is a genetic model of obesity which resulted from a spontaneous mutation in the cross between the Merck Stock M and Sherman rats (Zucker and Zucker, 1961; Bray, 1977). The obesity gene (fa) is transmitted as an autosomal Mendelian recessive trait. Rats which are homozygous for the fa gene are phenotypically obese with an onset detectable visually at 5 weeks of age (Josep, 1989). While the molecular mechanism of the obesity gene is unknown at present, the obesity syndrome closely resembles that displayed by juvenile onset obesity in humans as the fatty Zucker rat is characterized by hyperphagia and hyperlipidemia (Bjoumtorp and Sjostrom, 1971). The fatty rat is also used as a model of insulin resistance since it is glucose intolerant, and hyperinsulinemic, effects also noted in maturity onset diabetes.

Insulin Resistance and Glucose Homeostasis

Insulin resistance, as shown by lower whole body glucose utilization in euglycemic hyperinsulinemic clamp experiments, has been shown in fatty Zucker rats (Terrettaz and Jeanrenaud, 1983; Pénicaud et al., 1987). It was demonstrated that insulin resistance shown in vivo was attributed to both peripheral insulin resistance and impaired insulin action in inhibiting hepatic glucose production (Terrettaz and Jeanrenaud, 1983; Terrettaz et al., 1986). Assessments of individual tissues show that insulin binding was decreased in the liver (Mahmood et al., 1978), skeletal muscle (Crettaz et al., 1980), and adipose cells (Cushman et al., 1978). Insulin resistance in skeletal muscle has been well documented in vitro (Czech et al., 1978; Crettaz et al., 1980; Sherman et al., 1988) and in vivo (Pénicaud et al., 1987). As shown in vitro, the impaired response to insulin in skeletal muscle was characterized by defective glucose transport and glucose metabolism. Adipose tissue, on the other hand, does not seem to contribute to insulin resistance in fatty Zucker rats in the early stage as higher
glucose metabolism and glucose transport in response to insulin has been shown in isolated adipocytes (Czech et al., 1978). In older rats (>12 weeks of age), insulin resistance in adipose tissue becomes apparent as shown both in vivo (Pénicaud et al., 1987) and in vitro (Cushman et al., 1978). Probably as a consequence of reduced peripheral tissue glucose utilization in the insulin resistant state, glucose homeostasis in the fatty Zucker rat is perturbed. Although glucose tolerance was found to be normal or slightly abnormal when tested by intravenous (Crettaz et al., 1983; Ionescu et al., 1985) and intraperitoneal glucose administration (Becker-Zimmermann et al., 1982), glucose tolerance was shown to be markedly impaired when tested by oral ingestion of glucose, a more physiological method (Ionescu et al., 1985; Rohner-Jeanrenaud et al., 1986). In addition, impaired glucose tolerance was shown to progress with the age of the animal (Ionescu et al., 1985; Apweiler and Freund, 1993), similar to the development of insulin resistance in adipose tissue. The mechanism of abnormal glucose tolerance was shown to be at least partly due to high hepatic glucose production (Rohner-Jeanrenaud et al., 1986).

**Cardiac Insulin Resistance in the Fatty Zucker Rat**

Cardiac insulin resistance in fatty Zucker rats, as shown by lower glucose metabolism in isolated heart, was first documented by Zaninetti et al. (1983). The depressed glucose metabolism was shown to be associated with impaired glucose transport as measured by in vitro 3-O-methyl glucose efflux in the basal state and insulin-stimulated state. In the basal state, there was a 4 fold decrease in the glucose transport rate. When the isolated heart was perfused with increasing concentrations of insulin, glucose transport was responsive to insulin. However, the maximal response was only 50% of the lean control. Hence it was reported that there was a decrease in the insulin responsiveness in the fatty Zucker rat heart (Zaninetti et al., 1983). Similar findings were reported in another study using isolated cardiac myocytes (Eckel et al., 1985). It was shown that glucose transport was responsive to insulin but the maximal
response to insulin was lower than that found in the lean control. Eckel et al. (1985) also showed from Scatchard analysis that there was a decrease in the low affinity insulin binding sites on cardiac myocytes of fatty rats. This was shown by an unaltered binding of insulin at low insulin concentrations and a decrease in the binding of insulin at high concentrations of the hormone. However, it was determined that even with the same amount of insulin bound, the insulin action on glucose transport was significantly reduced in cardiac myocytes of obese rats. Thus altered insulin binding could not fully explain the defects in glucose transport. From the same study, it was also shown that insulin receptor internalization, as estimated from the lysosomal degradation of internalized insulin in chloroquine-treated cells, was reduced by 70% in cardiac cells of fatty Zucker rats (Eckel et al., 1985). However, this technique of insulin internalization estimation does not take into account the contribution from nonlysosomal degradation of insulin. Hence it could not provide an accurate estimation of the contribution of defective insulin internalization to impaired insulin action in the insulin resistant heart. Thus, possible defects at the level of the glucose transport system in cardiac insulin resistance in the fatty Zucker rat could not be ruled out.

**Cardiac Function of Fatty Zucker Rats**

Several studies have been performed to assess the mechanical function of the fatty Zucker rat heart. Impaired myocardial function was not apparent in the insulin resistant rat. Perfused heart experiments showed that the obese rat heart had a higher ventricular pressure-generating ability when compared to age-matched lean controls (Paradise et al., 1985; Rösen et al., 1986a). However, it was questionable whether the enhanced cardiac performance was due to increased ventricular mass. In fact, it was shown that the wall stress of the left ventricle at peak systole was reduced, reflecting ventricular dilation and/or decreased contractility (Paradise et al., 1985). In addition, it has been shown under hypoxic conditions that cardiac function in the fatty Zucker rat was depressed (Segel et al., 1980). It was shown
in obese rats of 19 weeks, but not 9 weeks of age, that the depressed heart function was associated with decreased stroke work and power, and diminished response to dobutamine in hypoxia (Segel et al., 1980). Thus under conditions of imposed stress, such as during exercise, depressed performance of the insulin resistant heart may become apparent.

IV) Experimental Rationale and Objectives

The glucose transport system in the fatty Zucker rat heart has been investigated in experiments using isolated hearts in vitro. It was shown that the total glucose transporter protein, as estimated by cytochalasin B binding studies, was depressed in isolated membrane fractions of the fatty Zucker rat heart (Zaninetti et al., 1989) suggesting the lower abundance of glucose transporters in the insulin resistant heart. From the same study it was also shown that perfusion of the isolated heart with insulin was able to induce glucose transporter mobilization to the plasma membrane fraction from the intracellular pool. Thus it was shown that glucose transporter translocation is functional in the fatty Zucker rat heart. However since cytochalasin B binding cannot distinguish between the Glut1 and Glut4 isoform, the results from this study could not actually determine the translocation of the Glut4 isoform.

The present project was aimed at studying the translocation of the insulin-regulated glucose transporter (Glut4) in the heart of the insulin resistant fatty Zucker rat under in vivo conditions. The interest in performing this study originates from the interest in studying the effect of vanadium, an insulin-mimetic agent, on Glut4 translocation in the fatty Zucker rat heart. Previous experiments have shown that vanadium can enhance the effect of insulin on glucose oxidation in isolated cardiac myocytes (Rodrigues et al., 1995). In addition, it has been shown in vitro that vanadium can induce Glut4 translocation in adipocytes (Pâquet et al., 1992). Moreover, Brichard et al. (1992) have shown that vanadium treatment in fatty Zucker rats improved glucose tolerance and ameliorated insulin resistance (Brichard et al., 1992).
was also shown by hyperinsulinemic euglycemic clamp experiments that the effect of vanadium treatment was associated with improved glucose metabolism in various tissues, with the most profound effect in the heart occurring without changing the expression of Glut4 (Brichard et al., 1992) suggesting the possible enhancement of cardiac Glut4 translocation brought about by vanadium treatment. Hence it is of interest to investigate the \textit{in vivo} effect of vanadium on Glut4 translocation in response to insulin. The present study was aimed at characterizing the cardiac Glut4 translocation response in the fatty Zucker rat by studying the effect of insulin and the time course of the translocation response \textit{in vivo}. It was hoped that the information obtained would be useful in designing future experiments to examine the effect of vanadium treatment on cardiac Glut4 translocation in fatty Zucker rats.

The specific objectives of the present study were to determine the effect of insulin dose and the time course of cardiac Glut4 translocation \textit{in vivo} in both lean and fatty Zucker rats by determining the subcellular distribution of Glut4. Glut4 content in the plasma membrane and intracellular membrane purified from cardiac tissue by subcellular fractionation was determined using a competitive ELISA method. The effect of insulin dose on cardiac Glut4 redistribution was studied using three doses of insulin and followed for 30 minutes after insulin injection.
MATERIALS AND METHODS

I) Chemicals and Materials

Subcellular Fractionation of Frozen Heart Ventriles

Tris[hydroxymethyl]aminomethane (Tris), imidazole (grade I), sodium pyrophosphate (tetrasodium salt, anhydrous), and phenylmethylsulfonylfluoride (PMSF) were obtained from Sigma Chemical Company (St. Louis, MO). KCl, and NaCl were obtained from BDH Inc. (Toronto, Ont.). Sucrose was manufactured by E. Merck Company (Germany) and purchased from VWR Scientific (Edmonton, Ab).

Na⁺/K⁺ ATPase Marker Enzyme Assay

NaCl, KCl, MgCl₂, trichloroacetic acid (TCA), H₂SO₄, and ammonium molybdate were obtained from BDH Inc. (Toronto, Ont). Ethylenediaminetetraacetic acid (EDTA, disodium salt, dihydrate), and ferrous sulfate (heptahydrate) were purchased from Sigma Chemical Company (St. Louis, MO).

Glut 4 Enzyme-Linked Immunosorbant Assay (ELISA)

KH₂PO₄ (monobasic), sodium carbonate (anhydrous), H₂SO₄, and hydrogen peroxide were purchased from BDH Inc. (Toronto, Ont). Polyoxyethylenesorbitan monolaurate (Tween 20), citric acid (monohydrate), citric acid (trisodium dihydrate), bovine serum albumin (BSA, ELISA grade) and 0-phenylenediamine dihydrochloride were obtained from Sigma Chemical Company (St. Louis, MO). Na₂HPO₄ (dibasic, anhydrous), and sodium bicarbonate were obtained from Fisher Scientific (Vancouver, B.C.). Skim milk powder (Carnation Inc., Toronto, Ont) was purchased from a local supermarket. Anti-Glut 4 monoclonal antibody, synthetic Glut4 C-terminus, and goat serum were obtained from East
Acres Biologicals (Southbridge, MA). Horseradish peroxidase-linked anti-mouse antibody was obtained from Amersham Life Science (Oakville, Ont).

II) Animals

Lean (Fa/?) and fatty (fa/fa) Zucker rats were obtained from the animal care unit of the Department of Physiology, UBC, and from the Animal Care Unit of the Vancouver Hospital, UBC site. Both colonies of Zucker rats originated from the same breeding colony in Charles River. Lean and fatty rats of both sexes and 19-21 weeks of age were used in the experiment. After an overnight-fast, the animals were anesthetized using sodium pentobarbital (65 mg/kg, i.p.). Regular insulin (Humulin®, Eli Lilly, Toronto, Ont) was given at various doses (2.5, 5, and 10 U/kg) by tail vein injection. After 5, 10, 15, 20, and 30 minutes, the chest cavity was opened and 1 ml of blood was withdrawn from the heart using a syringe. The heart was immediately removed and washed in distilled water to remove blood. After the removal of the aorta and atrial tissue, the ventricle was cut open, blotted dry and freeze-clamped in liquid nitrogen and stored at -70°C until use for subcellular fractionation.

III) Blood Sample Analyses

Blood collected from the heart was centrifuged for 15 minutes at 15,000 rpm using a desktop centrifuge (Biofuge 17R, model 2752, Baxter Scientific) to separate plasma. Plasma samples were stored at -70°C until the time of glucose and insulin determination. Glucose was determined using a glucose assay kit obtained from Boehringer Mannheim (Laval, Que). Plasma insulin was quantitated using an insulin RIA kit (Linco, St. Louis, MO) with a guinea pig anti-rat insulin antibody and rat insulin standards.
IV) Subcellular Fractionation of Frozen Heart Ventricles

The fractionation procedure was a modification of the method of Mansier et al. (1983) used to isolate highly purified sarcolemmal membrane using fresh heart ventricles. A schematic diagram of the fractionation procedure is illustrated in Figure 2. Frozen heart ventricles were powdered in a mortar cooled with liquid nitrogen and then transferred to centrifuge tubes containing 8 ml of buffer I which contained 10 mM Tris, 20 mM sodium pyrophosphate, and 0.1 mM phenylmethylsulfonylfouride (PMSF) at pH 7.8. The mixture was then homogenized using a polytron with a PT 10-35 probe (Brinkman Instruments Ltd, Toronto, Ont) at maximum speed with 2 to 3 bursts. Subsequently, the heart homogenate was subjected to hypotonic lysis by stirring on ice for 20 minutes. Hypotonic lysis was stopped by adding 2 ml of a solution containing concentrated KCl (600 mM), NaCl (150 mM) and sucrose (1250 mM) which brought the final concentration in the homogenate to 120 mM KCl, 30 mM NaCl, and 250 mM sucrose. The subsequent isolation of membrane fractions was done by differential centrifugation. All low-speed spins (≤31,000xg) were done using the Beckman J2-21 centrifuge and JA-17 rotor unless specified otherwise. After the hypotonic lysis, the mixture was centrifuged for 15 min at 500 x g to remove ventricular tissue which had not been broken down in the homogenization procedure. Four hundred microliters of the supernatant was collected as homogenate in several aliquots and then quickly frozen in liquid nitrogen. The remaining supernatant was made up to 15 ml with buffer II which contained 30 mM imidazole, 120 mM KCl, 30 mM NaCl, 20 mM sodium pyrophosphate, 0.1 mM PMSF, and 250 mM sucrose (pH 6.8). After a 20-minute spin at 7,000 x g, the supernatant was centrifuged for another 40 min at 31,000 x g. The resultant pellet was collected for the further purification of sarcolemmal membrane whereas the supernatant was set aside for isolation of intracellular membrane. The pellet was resuspended in 1 ml of buffer II using the homogenizer and then layered on top of 4 ml of 33% sucrose solution containing 300 mM KCl, 50 mM sodium pyrophosphate and 100 mM Tris at pH 7.4. After ultracentrifugation at
90,000 x g for 90 minutes using the Beckman L2-65B ultracentrifuge and SW 41 rotor, sarcolemmal membrane, seen as an opaque band on top of the 33% sucrose cushion, was collected and subsequently washed in wash buffer (30 mM imidazole, and 120 mM KCl, pH 6.8). After ultracentrifugation at 31,000 x g for 30 minutes using the Beckman L2-65B ultracentrifuge and Ty 65 rotor, sarcolemmal membrane was pelleted and then resuspended in 200 µl of buffer III (120 mM KCl, 250 mM sucrose, and 30 mM imidazole, pH 6.8). The supernatant, which was set aside for isolation of intracellular membrane, was ultracentrifuged at 150,000 x g for 60 min using the Beckman L5-50 ultracentrifuge and Ti 50.2 rotor. Intracellular membrane was further purified from the resultant pellet using a discontinuous sucrose gradient containing 4 ml of 33% sucrose and 4 ml of 50% sucrose with the same ingredients as the sucrose cushion as mentioned above. The sucrose gradient was centrifuged at 125,000 x g for 90 min using the Beckman L5-50 ultracentrifuge and SW 41 rotor. Intracellular membrane was obtained from the 33% sucrose layer first by washing using the wash buffer and then pelleted down by centrifugation at 65,000 x g for 30 minutes using the Beckman L5-50 ultracentrifuge and Ty 65 rotor. All fractions were resuspended in 200 µl of buffer III using the homogenizer and quickly frozen in liquid nitrogen. The fractions collected were stored at -70°C until further analysis.
Powder Frozen Heart Ventricle
Homogenize using polytron (max speed, 2-3 bursts)
hypotonic lysis
add conc. salt and sucrose
500xg (15 min)

P discard

Aliquot S dilute with buffer II
7,000xg (15 min)

P S discard

31,000xg (40 min)

P

33%

90,000xg (90 min)

S

150,000xg (60 min)

P discard

33%

50%

125,000xg (90 min)

33%

50%

65,000xg (30min)

Intracellular membrane

Plasma membrane

Figure 2. Subcellular fractionation of frozen heart ventricles
V) **Ouabain-sensitive Na\(^+\)/K\(^+\) ATPase Marker Enzyme Assay**

Ouabain-sensitive Na\(^+\)/K\(^+\) ATPase was used as a marker enzyme for sarcolemmal membrane. The activity was measured in all fractions obtained to examine possible contamination of sarcolemmal membrane in other subcellular fractions. Na\(^+\)/K\(^+\) ATPase activity was assayed by measuring the hydrolysis of non-radioactive ATP according to the method of Pierce and Dhalla (1983). Total ATPase activity was assayed in 1 ml of total assay volume containing Tris-HCl (50 mM), NaCl (100 mM), KCl (20 mM), MgCl\(_2\) (4 mM), EDTA (1 mM), and ATP (4 mM) at pH 7.4. Ouabain-inhibited ATPase activity was assayed in the same buffer with the presence of 2 mM ouabain (pre-dissolved in water with 10% [v/v] methanol). Na\(^+\)/K\(^+\) ATPase activity was calculated as the difference between total and ouabain-inhibited ATPase activity. The reaction was conducted at 37°C in a water bath for 10 minutes. The reaction was started by adding ATP to the reaction tubes and terminated by adding 1 ml of cold 12% (w/v) trichloroacetic acid (TCA) and subsequently putting the reaction tubes onto ice. Subsequently, proteins in the reaction mixture were removed by centrifugation at 3500 rpm for 15 minutes using the Beckman J-6B centrifuge and JS-4.2 rotor. Inorganic phosphate released from the hydrolysis of ATP in the reaction medium was chemically assayed according to the method of Taussky and Shorr (1952). In the chemical reaction, inorganic phosphate forms phosphomolybdic acid with ammonium molybdate. The phosphomolybdic acid is further reduced by ferrous sulphate to form a blue color which can be detected spectrophotometrically. After centrifugation to remove proteins, 75 μl of the reaction medium from each reaction tube was pipetted in duplicates into a 96-well plate (Evergreen Scientific, Los Angeles, CA). Inorganic phosphate was assayed by adding 50 μl of the ferrous sulphate-ammonium molybdate reagent which contained 1% (w/v) ammonium molybdate and 5% (w/v) ferrous sulphate in 1N sulphuric acid. A potassium acid phosphate (KH\(_2\)PO\(_4\)) solution (0 mM-0.86 mM) was used as standards. Color development was
allowed for 15 minutes after which absorbance was measured using a microplate reader (Model EL 309, Bio-Tek Instruments) at a wavelength of 750 nm. Enzyme activity was expressed as μmoles of inorganic phosphate released per hour per mg protein. Protein content of all fractions was quantitated using a commercial protein assay kit modified from the Lowry method (Bio-Rad Laboratories, Mississauga, Ont.)

VI) Glut 4 Competitive Enzyme-Linked Immunosorbant Assay (ELISA)

Glut4 protein levels in membrane fractions were quantitated using a competitive ELISA assay which consisted of four steps: blocking, primary antibody incubation, secondary antibody incubation, and substrate incubation. Between each step, the reaction plate (96-well maxisorp immunoplates [Nunc]) was washed and then dried by inverting the plate on paper towel. Each wash consisted of filling each well of the plate with wash buffer (KBS with 0.1% [v/v] Tween 20, pH 6.9) and then shaking the plate manually for 15 seconds. One day before the assay, the reaction plate was coated with a synthetic peptide consisting of the last 12 amino acid sequence of the Glut4 C-terminus. Fifty microliters of the synthetic peptide at a concentration of 0.125 mg/ml in 0.1 M carbonate buffer (pH 9.3) was pipetted into each well and then incubated overnight in a 50°C oven. After overnight incubation, the reaction plate was washed three times with wash buffer and then subjected to blocking with 10% heat inactivated goat serum for 2 hours at 37°C. Heat inactivation of goat serum was done in advance by incubating at 56°C for 30 minutes. During the blocking period, membrane fraction samples were first solubilized by adding Triton X-100 to a final concentration of 1% and then diluted with KBS containing 1% Triton X-100 in a low affinity binding plate (Evergreen Scientific). Just prior to the end of the blocking period, 30 μl of the monoclonal antibody (diluted 1:3000 in KBS containing 3% [w/v] skim milk, 1% [w/v] BSA [ELISA grade], and 0.05% [v/v] Tween 20) was added to 35 μl of the membrane samples and the internal standard in the low affinity plate. The plate was then mixed manually for a few
seconds. Subsequently, 50 μl of the mixture was transferred to the reaction plate after washing the plate 4 times. Incubation with the monoclonal antibody was conducted for two hours after which the reaction plate was washed 4 times and then subjected to incubation with the secondary antibody. One hundred microliters of the secondary antibody (horseradish peroxidase-linked anti-mouse antibody, diluted 1:1000 in KBS containing 1.5% (w/v) skim milk, 0.5% (w/v) BSA (ELISA grade), and 0.05% (v/v) Tween 20) was added to each well of the reaction plate. After incubation with the secondary antibody for 2 hours, the reaction plate was washed 3 times and then incubated with the substrate (20 mg 0-phenylenediamine dihydrochloride in 50 ml of 0.1 M citrate buffer [pH 5.0]). Three minutes before the addition of the substrate, 20 μl of H₂O₂ was added to 50 ml of the substrate solution. The plate was incubated with 150 μl of the substrate solution in each well for 20 minutes. The enzymatic reaction was stopped by adding 40 μl of 8 N sulphuric acid to each well. Color development was allowed for 10 minutes before absorbance readings were taken at 490 nm using an automated microplate reader (Model EL 309, Bio-Tek Instruments). Glut4 content was determined as per milligram membrane protein and the relative amount was obtained by comparing with an internal standard (heart homogenate of control Wistar rats) which was run in every assay. Protein content of all fractions was quantitated using a commercial protein assay kit modified from the Lowry method (Bio-Rad Laboratories, Mississauga, Ont.)

VII) Statistical Analysis

Results are presented as mean ± standard error of the mean. Formal statistical analysis was performed using the Number Cruncher Statistical Systems (NCSS 6.0) computer program. Two-way ANOVA was performed for experiments where the response due to two independent variables were studied and one-way ANOVA was performed when one independent variable was studied. The Newman-Keuls multiple comparison test was
performed for comparison of group means once the null hypothesis was rejected. A probability of $p<0.05$ was taken as the level of statistical significance.
RESULTS

I) Plasma Insulin Levels After Insulin Injection

Plasma insulin levels were determined in the basal state as well as in the insulin-stimulated state in lean and fatty Zucker rats. Tables 2 to 4 show the insulin values in the basal state and at various time points within a 30-minute period after insulin injection at doses 2.5, 5, and 10 U/kg to both phenotypes. In the basal state, plasma insulin level was four times higher in fatty Zucker rats as compared to the lean control (6.8 ± 1.4 ng/ml vs 1.5 ± 0.4 ng/ml). Insulin injection at a dose of 2.5 U/kg increased plasma insulin levels in both lean and fatty rats although the increase did not reach statistical significance in the lean phenotype (p>0.05, one-way ANOVA). Insulin injection at doses of 5 and 10 U/kg significantly increased plasma insulin levels in both phenotypes (p<0.05, one-way ANOVA).

II) Effect of Insulin Injection on Plasma Glucose levels

The acute plasma glucose responses to intravenous insulin injection were determined in both lean and fatty Zucker rats. Figure 3 shows the effect of insulin at a dose of 2.5 U/kg on plasma glucose levels in lean and fatty Zucker rats at various time points within a 30-minute period post insulin injection. In the basal state, fasting plasma glucose levels were similar in lean (7.38 ± 0.39 mM) and fatty Zucker rats (8.05 ± 0.67 mM). In lean Zucker rats, insulin injection at a dose of 2.5 U/kg significantly lowered the glucose level from the basal level at 10, 15, 20, and 30 minutes post insulin injection (p<0.05, one-way ANOVA followed by Newman-Keuls multiple comparison test). At 30 minutes after insulin injection, plasma glucose dropped to the lowest level of 2.63 ± 0.23 mM. In fatty Zucker rats, 2.5 U/kg insulin did not significantly lower plasma glucose at any of the time points post insulin injection (p>0.05, one-way ANOVA).
Table 2. Plasma insulin levels (ng/ml) of lean and fatty Zucker rats at various time points after insulin injection at a dose of 2.5 U/kg

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>5 min</th>
<th>10 min</th>
<th>15 min</th>
<th>20 min</th>
<th>30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean</td>
<td>1.5±0.4</td>
<td>1531±868</td>
<td>395±176</td>
<td>411±123</td>
<td>78±21</td>
<td>41±6.7</td>
</tr>
<tr>
<td></td>
<td>(7)</td>
<td>(6)</td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
</tr>
<tr>
<td>Fatty</td>
<td>6.8±1.4</td>
<td>609±160</td>
<td>1181±518*</td>
<td>472±170</td>
<td>523±255</td>
<td>128±36</td>
</tr>
<tr>
<td></td>
<td>(5)</td>
<td>(5)</td>
<td>(4)</td>
<td>(4)</td>
<td>(5)</td>
<td>(4)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± s.e.m. The number of animals in each group is indicated in parenthesis. * indicates significant difference when compared to the basal level of each phenotype using one-way ANOVA (p<0.05) followed by Newman-Keuls multiple comparison test.
Table 3. Plasma insulin levels (ng/ml) of lean and fatty Zucker rats at various time points after insulin injection at a dose of 5 U/kg

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>5 min</th>
<th>10 min</th>
<th>15 min</th>
<th>20 min</th>
<th>30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean</td>
<td>1.5±0.4</td>
<td>1563±302*</td>
<td>797±181</td>
<td>416±93</td>
<td>226±75</td>
<td>169±28</td>
</tr>
<tr>
<td></td>
<td>(7)</td>
<td>(5)</td>
<td>(6)</td>
<td>(5)</td>
<td>(6)</td>
<td>(5)</td>
</tr>
<tr>
<td>Fatty</td>
<td>6.8±1.4</td>
<td>583±456</td>
<td>551±236</td>
<td>1298±618*</td>
<td>653±137</td>
<td>374±58</td>
</tr>
<tr>
<td></td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± s.e.m. The number of animals in each group is indicated in parenthesis. * indicates significant difference when compared to the basal level of each phenotype using one-way ANOVA (p<0.05) followed by Newman-Keuls multiple comparison test.
Table 4. Plasma insulin levels (ng/ml) of lean and fatty Zucker rats at various time points after insulin injection at a dose of 10 U/kg

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>5 min</th>
<th>10 min</th>
<th>15 min</th>
<th>20 min</th>
<th>30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean</td>
<td>1.5±0.4</td>
<td>2128±709*</td>
<td>1481±225*</td>
<td>893±122</td>
<td>491±108</td>
<td>75±26</td>
</tr>
<tr>
<td></td>
<td>(7)</td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
</tr>
<tr>
<td>Fatty</td>
<td>6.8±1.4</td>
<td>3684±2265*</td>
<td>2359±660</td>
<td>889±421</td>
<td>832±245</td>
<td>1096±245</td>
</tr>
<tr>
<td></td>
<td>(5)</td>
<td>(3)</td>
<td>(5)</td>
<td>(5)</td>
<td>(5)</td>
<td>(4)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± s.e.m. The number of animals in each group is indicated in parenthesis. * indicates significant difference when compared to the basal level of each phenotype using one-way ANOVA (p<0.05) followed by Newman-Keuls multiple comparison test.
The acute effect of a higher insulin dose (5 U/kg) on plasma glucose levels of lean and fatty Zucker rats was investigated in the same time period. Plasma glucose levels in response to insulin injection at a dose of 5 U/kg is shown in Figure 4 for both phenotypes. In lean Zucker rats, plasma glucose was significantly lowered from the basal level at all time points following insulin injection (p<0.05, one-way ANOVA followed by Newman-Keuls multiple comparison test). In fatty Zucker rats, the higher insulin dose was not effective in lowering plasma glucose. Plasma glucose levels at all time points after insulin injection were not significantly different from the basal level in fatty rats (p>0.05, one-way ANOVA).

The acute effect of the highest insulin dose (10 U/kg) on plasma glucose levels was investigated in both lean and fatty Zucker rats and is shown in Figure 5. In lean Zucker rats, the effect of 10 U/kg insulin on plasma glucose was similar to that of 5 U/kg insulin. At all time points after insulin injection, plasma glucose was significantly lower than the basal level (p<0.05, one-way ANOVA followed by Newman-Keuls multiple comparison test). In fatty Zucker rats, plasma glucose did not decrease at any time point after insulin injection (p>0.05, one-way ANOVA), similar to the effect of the lower doses of insulin.
Figure 3. Effect of insulin injection (2.5 U/kg, iv) on plasma glucose levels in lean and fatty Zucker rats at various time points after insulin injection. Results are presented as mean ± s.e.m. Statistical analysis was performed using one-way ANOVA (p<0.05). * indicates significant difference when compared to the basal level using Newman-Keuls multiple comparison test. n≥4 in each group for each time point.
Figure 4. Effect of insulin injection (5 U/kg, iv) on plasma glucose levels in lean and fatty Zucker rats at various time points after insulin injection. Results are presented as mean ± s.e.m. Statistical analysis was performed using one-way ANOVA (p<0.05). * indicates significant difference when compared to the basal level using Newman-Keuls multiple comparison test. n≥4 in each group for each time point.
Lean | Fatty

Plasma glucose (mM)

Time after insulin injection (min)

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatty</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Indicate significant difference
Figure 5. Effect of insulin injection (10 U/kg, iv) on plasma glucose levels in lean and fatty Zucker rats at various time points after insulin injection. Results are presented as mean ± s.e.m. Statistical analysis was performed using one-way ANOVA (p<0.05). * indicates significant difference when compared to the basal level using Newman-Keuls multiple comparison test. n≥4 in each group for each time point.
Fatty

Time after insulin injection (min)

Plasma glucose (mM)

Lean

Fatty

0 5 10 15 20 30

Time after insulin injection (min)
III) Cardiac Glut4 Mobilization in Response to Insulin at a Single Time Point

Cardiac Glut4 mobilization to the plasma membrane in response to insulin, as indicated by an increase in the Glut4 content from the basal state, was investigated in lean and fatty Zucker rats. Figure 6 shows the plasma membrane Glut4 content in the basal state and at 20 minutes after insulin injection (10 U/kg) in both lean and fatty Zucker rat heart. In the basal state, plasma membrane Glut4 content was significantly lower in the fatty rat heart (8.74 ± 1.08 units) when compared to the lean control (13.19 ± 0.78 units) (p<0.05, two-way ANOVA). In the insulin-stimulated state, Glut4 mobilization to the plasma membrane was shown by a 30% and 39% increase in plasma membrane Glut4 content in the lean and fatty rat heart respectively. The elevation in Glut4 content in both phenotypes was statistically significant when compared to the basal level of each group (p<0.05, two-way ANOVA). In the fatty Zucker rat, insulin caused an increase in Glut4 content in the plasma membrane, but the level was significantly lower than the lean control in the insulin-stimulated state (p<0.05, two-way ANOVA).

Glut4 mobilization from the intracellular pool in response to insulin, as indicated by a decrease in Glut4 content from the basal level, was also investigated in lean and fatty Zucker rats. Figure 7 shows the Glut4 content in the intracellular membrane in the basal state and after insulin stimulation for both phenotypes. In lean control rat hearts, insulin (10 U/kg) caused Glut4 mobilization from the intracellular membrane as shown by a 37% decrease in Glut4 content in the intracellular membrane (p<0.05, two-way ANOVA). Intracellular membrane Glut4 content in the basal state was significantly lower in fatty rat hearts (11.6 ± 1.24 units) as compared to the lean control (20.66 ± 2.39 units) (p<0.05, two-way ANOVA). In the insulin-stimulated state, Glut4 mobilization from the intracellular membrane in the fatty rat did not occur since there was no significant drop in the Glut4 content in response to insulin.
Figure 6. Plasma membrane Glut4 content in lean and fatty Zucker rat hearts in the basal state and in the insulin-stimulated state. Insulin was injected at a dose of 10 U/kg and the heart was collected at 20 minutes post insulin injection. Non-injected animals were used for the basal control. Plasma membrane was isolated from frozen ventricular tissue using the subcellular fraction method as outlined in Materials and Methods. Statistical analysis was performed using two-way ANOVA (p<0.05). * indicates significant difference when compared to the basal level and # indicates significant difference when compared to the lean control using Neuman-Keuls multiple comparison test.
Figure 7. Intracellular membrane Glut4 content in lean and fatty Zucker rat hearts in the basal state and in the insulin-stimulated state. Insulin was injected at a dose of 10 U/kg via the tail vein and the heart was collected at 20 minutes post insulin injection. Non-injected animals were used for the basal control. Intracellular membrane was isolated from frozen ventricular tissue using the subcellular fractionation method as outlined in Materials and Methods. Results are presented as mean ± s.e.m. Statistical analysis was performed using two-way ANOVA (p<0.05). * indicates significant difference when compared to the basal level and # indicates significant difference when compared to the lean control using Neuman-Keuls multiple comparison test.
IV) **Dose Effect of Insulin on Cardiac Glut4 Mobilization**

The effect of several insulin doses on cardiac Glut4 mobilization was examined in both the plasma membrane and intracellular membrane for both phenotypes of rats. Figure 8 shows plasma membrane Glut4 content at 20 minutes after insulin injection (2.5, 5, and 10 U/kg) to lean and fatty Zucker rats. For both phenotypes, all three insulin doses caused a significant increase in plasma membrane Glut4 content when compared to the basal level of each phenotype (p<0.05, one-way ANOVA). The dose effect of insulin was similar for each phenotype (1.2 to 1.3 fold increase in Glut4 content in the lean phenotype and 1.4 to 1.5 fold increase in the fatty phenotype). Glut4 content in the plasma membrane was similar irrespective of the insulin dose for lean Zucker rats (one-way ANOVA) as well as for fatty Zucker rats (one-way ANOVA).

The effect of insulin doses on Glut4 content in the intracellular membrane is shown in Figure 9 for both phenotypes. In the lean control, Glut4 mobilization from the intracellular membrane was responsive to all three insulin doses as shown by a significant decrease from the basal level (p<0.05, one-way ANOVA). The effect of all three insulin doses was similar as there was no significant difference among the doses (one-way ANOVA). In the fatty rat heart, all three insulin doses had no effect on Glut4 mobilization from the intracellular membrane. There were no significant differences in Glut4 content between the basal state and any of the insulin-stimulated states (one-way ANOVA).
Figure 8. Effect of insulin on plasma membrane Glut4 content in lean and fatty Zucker rat hearts. Insulin was injected at a dose of 2.5, 5, and 10 U/kg to lean and fatty Zucker rats. At 20 minutes after insulin injection, the heart was collected and stored frozen. Plasma membrane was isolated from frozen ventricular tissue using the subcellular fractionation method as outlined in Materials and Methods. Results are presented as mean of 5 to 7 rats for each insulin dose. Error bars are standard error of the mean. Statistical analysis was performed using one-way ANOVA (p<0.05). * indicates significant difference when compared to the basal level using Neuman-Keuls multiple-comparison test.
Lean -o- Fatty

Plasma membrane Glut4 content
(relative units)

Insulin dose (U/kg)

* indicates statistical significance.
Figure 9. Effect of insulin on intracellular membrane Glut4 content in lean and fatty Zucker rat hearts. Insulin was injected at a dose of 2.5, 5, and 10 U/kg to lean and fatty Zucker rats. At 20 minutes after insulin injection, the heart was collected and stored frozen. Intracellular membrane was isolated from frozen ventricular tissue using the subcellular fractionation method as outlined in Materials and Methods. Results are presented as mean of 5 to 7 rats for each insulin dose. Error bars are standard error of the mean. Statistical analysis was performed using one-way ANOVA (p<0.05). * indicates significant difference when compared to the basal level using Neuman-Keuls multiple-comparison test.
The graph shows the intracellular membrane Glut4 content (relative units) in response to different insulin doses (U/kg) for Lean and Fatty groups. The y-axis represents the intracellular membrane Glut4 content, while the x-axis represents the insulin dose in U/kg. The data points for Lean and Fatty groups are indicated by solid circles and open circles, respectively. The asterisks indicate statistically significant differences between the groups.
V) Temporal Effect of Insulin on Cardiac Glut4 Mobilization

The time course of Glut4 translocation in the heart of lean and fatty Zucker rats was studied at 5, 10, 15, 20, and 30 minutes after insulin injection. As shown in Figure 8 and 9, the effects of all three insulin doses on Glut4 translocation were similar. In addition, preliminary statistical analysis showed that there were no significant differences in Glut4 content at the various time points after insulin injection for each of the insulin dose used (one-way ANOVA). Therefore, the translocation responses obtained with each of the insulin dose were combined to construct the time course of the translocation response (Figure 10). Glut4 level in the plasma membrane was increased from the basal level of 13.19 ± 0.78 units and 8.74 ± 1.08 units to 16.51 ± 0.82 units and 12.84 ± 0.64 units for the lean and fatty phenotype respectively. In each phenotype, Glut4 mobilization to the plasma membrane was similar between 5 and 30 minutes after insulin injection. The level of Glut4 in the plasma membrane was not significantly different among all the time points after insulin injection (p>0.05, one-way ANOVA). A lower level of Glut4 in the plasma membrane was observed in the insulin-stimulated state for the fatty Zucker rat.

The time-dependent effect of insulin (combined doses) on Glut4 mobilization from the intracellular membrane fraction was examined and is shown in Figure 11 for both phenotypes. In lean rat hearts, Glut4 content in the intracellular fraction decreased from 20.66 units in the basal state (Figure 7) to 14.01 units at 5 minutes after insulin injection. There was no further Glut4 mobilization from the intracellular fraction beyond 5 minutes as shown by no significant differences among 5, 10, 15, 20, and 30 minutes after insulin injection (p>0.05, one-way ANOVA). In the fatty phenotype, intracellular membrane Glut4 content in the stimulated state was not significantly different (p>0.05, one-way ANOVA) among the various time points and was similar to the basal level (11.6 units ± 1.24, Figure 7).
Figure 10. Plasma membrane Glut4 content in lean and fatty Zucker rat hearts at various time points after insulin injection. The response at each time point was obtained by combining the data for all insulin doses (2.5, 5, 10 U/kg). Plasma membrane was isolated from frozen ventricular tissue using the subcellular fractionation method as outlined in Materials and Methods. Results are presented as mean ± s.e.m. The number of animals at each time point is indicated in parentheses. Statistical analysis was performed using one-way ANOVA (p<0.05) for each phenotype to compare the response among the various time points.
Figure 11. Intracellular membrane Glut4 content in lean and fatty Zucker rat hearts at various time points after insulin injection. The response at each time point was obtained by combining the data for all insulin doses (2.5, 5, 10 U/kg). Intracellular membrane was isolated from frozen ventricular tissue using the subcellular fractionation method as mentioned in Materials and Methods. Results are presented as mean ± s.e.m. The number of animals at each time point is indicated in parentheses. Statistical analysis was performed using one-way ANOVA (p<0.05) for each phenotype to compare the response among the various time points.
VI) Na\(^+\)/K\(^+\) ATPase Activity in Subcellular Fractions

The subcellular fractionation method used in the current study was evaluated by the use of a plasma membrane marker enzyme. Na\(^+\)/K\(^+\) ATPase was selected as the plasma membrane marker enzyme and was measured in the various fractions obtained from Wistar, lean and fatty Zucker rat hearts (Table 5). As shown by fractions obtained from Wistar rats, the plasma membrane fraction was 88 times more enriched in Na\(^+\)/K\(^+\) ATPase activity when compared to the homogenate. There was little plasma membrane contamination in the intracellular membrane. Only 15% of the Na\(^+\)/K\(^+\) ATPase activity in the plasma membrane fraction was present in the intracellular membrane fraction. In lean Zucker rats, a similar Na\(^+\)/K\(^+\) ATPase activity was found in the plasma membrane as compared to Wistar rats. The marker enzyme activity in the intracellular membrane was 13% of the plasma membrane fraction. In fatty Zucker rats, Na\(^+\)/K\(^+\) ATPase activity in the plasma membrane was 60% of the lean control with 15% plasma membrane contamination in the intracellular membrane, similar to that seen in Wistar and lean Zucker rats.
Table 5. Na⁺/K⁺ ATPase activity (μmoles Pi/hr/mg) in subcellular fractions obtained from Wistar, lean and fatty Zucker rat hearts

<table>
<thead>
<tr>
<th></th>
<th>Wistar</th>
<th>Lean Zucker</th>
<th>Fatty Zucker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>1.3 ± 0.5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>n=6</td>
<td>n=11</td>
<td>n=10</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>114.9 ± 9.4</td>
<td>109.3 ± 7.7</td>
<td>66.2 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>n=11</td>
<td>n=9</td>
<td>n=10</td>
</tr>
<tr>
<td>Intracellular membrane</td>
<td>16.8 ± 2.6</td>
<td>14.0 ± 2.2</td>
<td>9.8 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>n=10</td>
<td>n=8</td>
<td>n=8</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± s.e.m. ND denotes not detected.
DISCUSSION:

I) Evaluation of Subcellular Fractionation Method

With the subcellular fractionation method used in the current study, an approximate 1.3 to 1.4 fold increase in Glut4 content in the plasma membrane was obtained with maximal insulin stimulation. The magnitude of cardiac Glut4 recruitment to the plasma membrane is consistent with the value reported by other biochemical studies of 1.4 fold to 3 fold increase in the heart (Uphues et al., 1995) and 1.4 to 2 fold in skeletal muscle (King et al., 1992; Galante et al., 1994; Napoli et al., 1995) in response to insulin in vivo. In the heart, the magnitude of the translocation response to the action of insulin is consistent with the magnitude of increase in glucose transport reported by Watanabe et al. (1984) and Wheeler (1988), but not consistent with the reports of others (Zaninetti et al., 1988; Zaninetti et al., 1989). It was suggested that the higher glucose transport response than transporter recruitment caused by insulin was due to a change in the functional properties of the glucose transporter on the cell surface (Zaninetti et al., 1988). Alternatively, it may be due to the limitations of subcellular fractionation techniques in estimating glucose transporter translocation. It has been shown by immunogold labeling studies that insulin caused a 40 fold increase in the amount of Glut4 in the plasma membrane in both the heart (Slot et al., 1991b) and adipose tissue (Slot et al., 1991a). In addition, a higher magnitude of Glut4 translocation could be directly estimated by photolabeling of glucose transporters on the cell surface (Calderhead et al., 1990; Holman et al., 1990). Both of these techniques do not involve the isolation of membrane fractions and may avoid cross contamination of subcellular membrane fractions. In spite of the limitations in fractionation techniques for the estimation of subcellular distribution of Glut4, it remains the most commonly used method since it is relatively easy to perform and can be used for whole tissue whereas cell-surface photolabeling can be done only with isolated cells. Whether translocation was induced in vivo or in vitro is also a contributing factor to the differences in
the magnitude of Glut4 translocation reported. In general, a higher degree of cardiac glucose transporter translocation, ranging from 2 to 8 fold, was estimated from *in vitro* experiments (Wheeler, 1988; Zaninetti et al., 1988; Zaninetti et al., 1989; Kolter et al., 1992; Sun et al., 1994).

The current fractionation method was also evaluated by measuring the activity of Na\(^+\)/K\(^+\) ATPase, a commonly used plasma membrane marker enzyme. As shown in Wistar rats, the plasma membrane fraction was highly enriched (88 fold) in marker enzyme activity when compared to the homogenate. Thus the plasma membrane fraction isolated from this fractionation procedure appeared to be relatively pure. It was also shown that plasma membrane contamination in the intracellular membrane was low (below 15%) in both Wistar and Zucker rats. As there is no currently available marker for the intracellular Glut4 which is mostly located in T-V elements, the relative enrichment of the intracellular membrane fraction could not be determined. However as shown in the present study, a 37% decrease in the Glut4 content in response to insulin stimulation confirmed that the intracellular membrane obtained closely represents the insulin-regulated pool as shown by immunogold labeling experiments (Slot et al., 1991b) and other biochemical studies (Zaninetti et al., 1989; Rodnick et al., 1992). In the present study, the possibility of intracellular Glut4 contamination in the plasma membrane cannot be ruled out due to the non-availability of a marker specific for the intracellular Glut4 compartment.

The Na\(^+\)/K\(^+\) ATPase activity of the plasma membrane in fatty Zucker rats was shown to be 40% lower than the lean control. This may indicate a difference in the purity of the plasma membrane fractions between the phenotypes. Alternatively, this may indicate an overall decrease in Na\(^+\)/K\(^+\) ATPase activity in the fatty Zucker rat heart. At present there are no reports on cardiac Na\(^+\)/K\(^+\) ATPase activity in the fatty Zucker rat. However, altered activity of the enzyme has been reported in the retina and in the liver of the fatty Zucker rat.
(Ottlecz et al., 1993; Ruiz-Montasell et al., 1994). Therefore it is not directly discernible from this study whether the plasma membrane fraction from both phenotypes were of the same purity. However, a 40% reduction of the enzyme activity of Na\(^+\)/K\(^+\) ATPase in the plasma membrane fraction of the fatty rat heart due to relatively more contamination with intracellular compartments is unlikely as the recruitment of Glut4 to this fraction in response to insulin was visible. If the plasma membrane fraction was truly contaminated with 40% more intracellular contaminants, the effect of insulin on transporter translocation would have been diluted and would probably have been undetectable. To confirm that the membrane fractions from both phenotypes are of the same purity, experiments with the use of other plasma membrane marker enzymes are necessary to further evaluate the purity of the membrane fractions. Alternative plasma membrane marker enzymes such as 5' nucleotidase and adenylate cyclase could be measured to compare the relative purity of the plasma membrane between lean and fatty Zucker rats (Evans, 1987). In addition, the use of negative marker enzymes to determine the relative contamination in the plasma membrane is another alternative and indirect method for determining the relative purity of the membrane fractions. Mitochondrial and sarcoplasmic reticular membrane are the most probable contaminating membranes in the plasma membrane fraction. Thus sodium azide-sensitive Na\(^+\)/K\(^+\) ATPase, a marker for mitochondrial membrane, and Ca\(^{2+}\) ATPase, a marker for sarcoplasmic reticulum, are possible negative marker enzymes that could be measured to evaluate relative contamination in the plasma membrane fractions.

II) **Insulin Resistance in the Fatty Zucker Rat**

In the present study, exogenous insulin was shown to significantly lower plasma glucose in lean but not fatty Zucker rats. This was not due to the lack of an increase in plasma insulin levels *in vivo* in the fatty group since the insulin level was significantly elevated in both phenotypes. These results are in agreement with previous findings that exogenous
insulin is ineffective in lowering plasma glucose in the fatty Zucker rat (Smith and Czech, 1983). It was shown that in eviscerated fatty Zucker rats, the rate of glucose disappearance in plasma was much less sensitive to exogenous insulin and that the maximal response was markedly reduced. Since the maximal response was reached at physiological concentrations of insulin (Smith and Czech, 1983), it can be speculated that the use of higher insulin doses will not likely to improve the response. Perturbed glucose homeostasis in the fatty Zucker rat has been shown to be due to both hepatic and peripheral insulin resistance (Terrettaz and Jeanrenaud, 1983; Terretaz et al., 1986; Rohner-Jeanrenaud et al., 1986). However, the inability of exogenous insulin to reduce plasma glucose observed in the present study was not likely due to the inability of insulin to shut down hepatic glucose output since the insulin doses used were more than sufficient to maximally suppress hepatic glucose production (Terrettaz et al., 1986). Therefore whole body insulin resistance observed in the fatty rat was probably due to peripheral resistance.

III) **Lower Expression of Cardiac Glut4 in the Fatty Zucker Rat**

The finding of a lower Glut4 content in the plasma membrane and intracellular fraction of the fatty rat heart in the present study suggests that there may be an overall decrease in the cardiac expression of Glut4. This is consistent with the *in vitro* study of Zaninetti et al. (1989) in which the total number of glucose transporters, as measured in the subcellular fractions by cytochalasin B binding, was estimated to be reduced by half in both the basal and insulin-stimulated state. However, since cytochalasin B binding cannot distinguish between Glut1 and Glut4, the contribution of Glut4 could not be estimated from this study. Lower cardiac expression of Glut4 in the fatty rat was also shown in another study which demonstrated a lower Glut4 content in the crude homogenate (Uphues et al., 1995). When measured in isolated membrane fractions, the Glut4 content was also reduced, although not to a significant degree in the plasma membrane. Hence from these two reports along with the
In the present study, there is consensus that the expression of Glut4 protein in the fatty rat heart is depressed. Interestingly, the gene expression of Glut4 in the fatty rat heart was shown to be enhanced as indicated by a higher level of mRNA (Petersen et al., 1991). This is probably due to the higher level of insulinemia in the obese rat since Glut4 gene expression was shown to be upregulated by insulin in isolated ventricular myocytes (Petersen et al., 1995). The higher expression of the Glut4 gene with a lower expression of the Glut4 protein could mean a lower translation of the mRNA in the insulin resistant heart. These results are not well understood at present and further experiments are necessary to elucidate the mechanism.

In the present study, an approximate 20% reduction in the plasma membrane Glut4 content was shown in the fatty rat heart as compared to the lean control in the maximally insulin-stimulated state. Such a reduction cannot fully account for the 50% decrease in glucose transport in response to maximal insulin stimulation when compared to the lean phenotype (Zaninetti et al., 1983; Eckel et al., 1985; Zaninetti et al., 1989). A 50% reduction in total glucose transporter content which was estimated previously using cytochalasin B binding (Zaninetti et al., 1989) was probably an overestimation since cytochalasin B binds to both the Glut1 and Glut4 isoform. As shown previously, the regulation of plasma membrane glucose transporter by insulin was perturbed (Zaninetti et al., 1989). It was shown that insulin stimulation decreased the Hill coefficient of cytochalasin B binding to the glucose transporters, indicating a change in the positive cooperativity amongst glucose transporters upon insulin stimulation. This may indicate defective activation of plasma membrane glucose transporters by insulin which may contribute partly to defective glucose transport in the insulin resistant heart. Another possible contributing factor of defective glucose transport in the fatty rat heart is a defective PKC. An activator of PKC, phorbol myristate acetate (PMA), was shown to induce a concentration-dependent increase in glucose transport in the lean Zucker rat heart while it had no effect in stimulating glucose transport in the fatty Zucker rat heart (Van de Werve et al., 1987). Detailed analysis of PKC in the heart of Zucker rats showed a
perturbed cellular distribution of PKC and defective translocation in response to PMA. Since PKC has been suggested to play a role in insulin signaling leading to glucose transporter translocation (Nishimura et al., 1994), defective PKC may also contribute to defective glucose transport in the fatty rat heart.

In the basal state, glucose transport was shown to be reduced in the fatty rat heart (Zaninetti et al., 1983; Zaninetti et al., 1989). In the present study, basal plasma membrane Glut4 content was shown to be reduced by 34% in the fatty rat heart. Although Glut4 is mostly located intracellularly in the basal state, the contribution of cell-surface Glut4 to basal glucose transport is not known. It is logical to speculate that the expression of the Glut1 isoform may also be decreased since a reduction in total glucose transporter (both Glut1 and Glut4) in the plasma membrane by more than 50% in the basal state has been reported by Zaninetti et al. (1989). Estimation of glucose transporter number specifically for the Glut1 isoform would be necessary to test this speculation.

The implication of cardiac Glut4 depletion on heart function in the fatty Zucker rat heart is not clear at present. Previous experiments have established an important role of defective glucose utilization in impaired cardiac function in animal models of diabetic cardiomyopathy (Rösen et al., 1986b; Nicholl et al., 1991). In the diabetic state, myocardial glucose transport was shown to be depressed as a result of glucose transporter depletion (Eckel and Reinaur, 1990; Camps et al., 1992; Garvey et al., 1993). Hence it was proposed that Glut4 loss may be an important determinant of myocardial dysfunction (Garvey et al., 1993). In the present study, the lower cardiac expression of Glut4 demonstrated in the fatty rat heart can only partly account for the diminished glucose transport response to insulin reported in the literature. Although cardiac function in the fatty Zucker rat was not impaired under normal conditions (Paradise et al., 1985; Rösen et al., 1986a), impaired heart function has been shown under hypoxic conditions (Segel et al., 1980). In the literature, there is
evidence that myocardial glucose utilization is increased under ischemic conditions (Opie et al., 1988) and that enhanced glucose utilization can protect the heart from postischemic injury (Owen et al., 1990; Mallet et al., 1990; Eberli et al., 1991). In normal conditions, fatty acid metabolism was shown to provide a large portion of the energy for cardiac performance in the fatty rat whereas it was of minor importance in the lean phenotype (Rösen et al., 1986a). It might be possible that cardiac dysfunction may become manifest in the obese Zucker rat when myocardial substrate metabolism shifts from fatty acid to glucose in hypoxic or ischemic states. Hence it may be relevant to assess myocardial substrate metabolism and transport, and cardiac performance under these conditions.

IV) **Dose Effect of Insulin on cardiac Glut4 Translocation**

Insulin in doses of 2.5-10 U/kg produced similar effects on cardiac Glut4 mobilization from the intracellular compartment to the plasma membrane in lean Zucker rats. In fatty Zucker rats, the magnitude of Glut4 recruitment to the plasma membrane was found to be similar to that in lean Zucker rats indicating Glut4 translocation was similarly responsive to insulin. The present experiment is the first to study the dose effect of insulin on cardiac Glut4 translocation *in vivo*. The inability of insulin to produce a dose-dependent effect on transporter translocation observed in the current study was probably because maximal response had been reached at 2.5 U/kg. At present, the dose effect of insulin on Glut4 translocation has been studied only in adipocytes and under *in vitro* conditions. It has been demonstrated by Karnieli et al. (1981) using cytochalasin B binding studies that total glucose transporter translocation was maximally stimulated at an insulin concentration of 0.7 nM which is equivalent to 100 μU/ml or about 6 ng/ml. In the current experiment, pharmacological concentrations of insulin were achieved with intravenous injection of the three insulin doses as shown by plasma insulin levels of at least 41 ng/ml, more than 20 times higher than the basal level in lean Zucker rats. In skeletal muscle, it has been shown that a
three fold elevation in plasma insulin by the administration of oral glucose caused an approximate 30% increase in Glut4 present in the plasma membrane (Napoli et al., 1995), similar to the magnitude of Glut4 recruitment shown in the current experiment. This further supports the present finding that the maximal translocation response was reached at an insulin dose of 2.5 U/kg. Hence higher insulin doses did not produce a dose-dependent effect in the translocation response. To investigate whether cardiac Glut4 translocation is equally sensitive to insulin in both phenotypes, a dose response curve with lower doses of insulin would be required. However, due to the variation in plasma insulin concentrations obtained from insulin injection, it would be difficult to control the amount of insulin stimulation \textit{in vivo} in a precise manner. In addition, with the small magnitude of translocation response obtained with subcellular fractionation, stimulation with lower doses of insulin may not produce detectable responses. Therefore, in order to investigate the effect of submaximal concentrations of insulin on cardiac Glut4 translocation, the use of an \textit{in vitro} preparation may be necessary.

V) Temporal Effect of Insulin on Cardiac Glut4 Translocation

It was demonstrated in the present study that, as early as 5 minutes after insulin injection, the translocation response was detectable in the plasma membrane as well as in the intracellular membrane in lean Zucker rats. The response was shown to persist throughout the 25-minute period without further translocation. The present experiment is the first to study the time course of \textit{in vivo} Glut4 translocation in the heart. The observation that maximal glucose transporter translocation was obtained as early as 5 minutes post insulin injection is in general agreement with the observation in isolated adipocytes (Karnieli et al., 1981) but not in 3T3-L1 adipocytes (Gibbs et al., 1988; Yang et al., 1992). It was demonstrated that maximal glucose transporter translocation response in isolated adipocytes, as shown by glucose transporter mobilization from the intracellular compartment to the plasma membrane, was reached following 5 minutes of insulin incubation (Karnieli et al., 1981). On the other hand,
the half-time in 3T3-L1 adipocytes was found to be 4 minutes by Gibbs et al. (1988) and 5.4 minutes by Yang et al. (1992), indicating maximal translocation response was obtained with insulin stimulation for 8 and 10.8 minutes respectively. The difference in the kinetics of the translocation response reported may be due to differences between isolated adipocytes and adipose cell lines. In the present study, it could not be determined whether maximal translocation occurred earlier than 5 minutes since the translocation response was not measured before this period. Karnieli et al. (1981) also showed that the redistribution of glucose transporter in response to insulin persisted for 45 minutes, or as long as the insulin was present in the incubation medium (Karnieli et al., 1981). In the present study, it was demonstrated that the translocation response was still present even at 30 minutes post insulin injection. This can be easily explained by the fact that plasma insulin levels were still elevated at this time. The finding that the distribution of Glut4 was still high in the plasma membrane and low in the intracellular membrane 30 minutes after insulin injection is also in agreement with the translocation of Glut4 in vivo shown in skeletal muscle (Napoli et al., 1995). It was shown that translocation was significantly stimulated at 30 and 60 minutes after an oral glucose load at which time plasma insulin levels were elevated by 3 to 4 fold.

In the fatty Zucker rat, the time profile of Glut4 recruitment to the plasma membrane was similar to the lean phenotype as shown by elevated Glut4 content in the plasma membrane which started at 5 minutes and persisted throughout the 25-minute period. Since maximal translocation was already reached at 5 minutes post insulin injection, the present study cannot determine whether the translocation response is equally rapid in both phenotypes. In order to obtain the time profile of the translocation response before maximal translocation was reached, precise control of the insulin stimulation period is required. This may be difficult to do in vivo since the removal of insulin stimulation from the heart requires several steps including the removal of the heart from the chest cavity and the removal of blood by rinsing. An alternative method would be to perform in vitro experiments using isolated cardiac
myocytes which would allow more precise control of the timing of insulin stimulation. In fatty Zucker rats, the time profile showed no translocation response in the intracellular membrane, as shown by the same Glut4 content in the basal state and throughout the entire 30-minute period post insulin injection. This further confirmed the observation that insulin was not able to mobilize Glut4 from this membrane fraction in the fatty rat heart.

VI) Cardiac Glut4 Translocation in the Fatty Zucker Rat

In the fatty Zucker rat, Glut4 recruitment to the plasma membrane was shown to be equally responsive to insulin when compared to lean Zucker rats, in terms of the fold difference as compared to the basal level. However, since the maximal translocation response had been reached with the lowest insulin dose and at the earliest time point, it cannot be determined from the present study whether the translocation response is equally sensitive to insulin in the two phenotypes and whether the time course of translocation is different. Nevertheless, the maximal translocation response was shown to be similar in both lean and fatty Zucker rats. However, since a lower basal level of Glut4 was observed, the absolute amount of plasma membrane Glut4 in the insulin-stimulated state was also lower than in the lean control (Figure 6). The finding that Glut4 recruitment to the plasma membrane was equally responsive in both phenotypes of rats is contrary to the finding of Uphues et al. (1995) who reported a diminished response of Glut4 recruitment to the plasma membrane when compared to the lean control. It was shown that insulin injection given intravenously caused a significant recruitment of Glut4 to the plasma membrane. However, there was a 3 fold increase in plasma membrane Glut4 content in the lean rat heart whereas there was only a 1.4 fold increase in the fatty phenotype. Thus the magnitude of translocation reported for the fatty rat is similar to the value reported in the present study while the magnitude for lean rats reported by Uphues et al. (1995) was much higher. The discrepancy may be due to a much higher dose of insulin (40 U/kg) used in the study of Uphues et al. (1995). However, since we
showed no further increases in translocation with insulin doses higher than 2.5 U/kg in both phenotypes of rats, this possibility is not likely. The discrepancy also cannot be explained by the possibility of the selective purification of plasma membrane in lean animals in the current study as shown by differences in the marker enzyme activity between the two phenotypes since such possibility would favor the results obtained by Uphues et al. (1995). Despite the fact that the fatty Zucker rat was shown to be insulin resistant as indicated by the inability of exogenous insulin to significantly lower plasma glucose, it was shown that Glut4 mobilization to the plasma membrane in the fatty rat heart was responsive to insulin. Since cardiac tissue only contributes a very small portion of total body weight, total body insulin resistance is probably not well reflected by cardiac tissue. Adipose tissue, which comprises ~45% of total body weight of the fatty rat over 14 weeks of age (Zucker and Antoniades, 1972), and skeletal muscle, which is a major site of glucose disposal as shown in human subjects (DeFronzo et al., 1985) would mostly account for and reflect whole body insulin resistance in the fatty Zucker rat.

It was also shown in the present study that Glut4 recruitment to the plasma membrane in the fatty rat heart was not associated with the mobilization of Glut4 from the intracellular pool. This cannot be explained by small sample size since this was shown to be the case with all insulin doses and at all time points studied. This finding is in contrast to the results of the study of Zaninetti et al. (1989) who reported a decrease in total glucose transporter in the microsomes in response to insulin. The controversy may be explained by the use of cytochalasin B binding employed for glucose transporter estimation in the study of Zaninetti et al. (1989) which could not distinguish between Glut1 and Glut4. The Glut4 results for the intracellular membrane are similar to those reported by Uphues et al. (1995) who showed that insulin did not significantly reduce Glut4 content in the intracellular membrane. There are several possible explanations for this finding. One is that the subcellular fractionation method employed in this study and in that of Uphues et al. selectively favored the isolation of the
insulin-regulated pool of Glut4 in lean Zucker rats but not in fatty rats. Consequently it may
be possible that the intracellular membrane obtained from fatty rat hearts was not the same
intracellular pool as obtained from lean rat hearts. The possibility of obtaining different
membrane fractions between two phenotypes of rats using the same fractionation procedure is
not certain. However it has been shown that the fatty acid composition of cardiac phospholipids isolated from fatty rat hearts differed significantly from that of the lean controls
(Wahle et al., 1991). This may possibly lead to differences in the physicochemical properties
of cell membranes in the heart of fatty rats and possible differences in the buoyant density of
the cell membranes. To confirm that the intracellular membrane fraction obtained from lean
and fatty rat hearts is from the same cellular location, detailed analysis of protein composition
of the membrane fractions may be necessary since there is no currently available marker
specific for the insulin-regulated pool of Glut4.

Alternatively, the unresponsiveness of intracellular Glut4 to insulin may be due to the
redistribution of Glut4 in the fatty rat heart as suggested by Uphues et al. (1995). In the
current study, it was shown that the basal plasma insulin level was 4 times higher in the fatty
Zucker rat. Hence it may be possible that in the basal state, Glut4 recruitment from the
intracellular store was already induced by elevated plasma insulin levels in the fatty rat thereby
causing depletion of Glut4 in the intracellular fraction. This can explain the lower intracellular
Glut4 content in the fatty rat heart in the basal state observed in present study as well as that
of Uphues et al. (1995). It was shown that rab4A, a small G-protein which was previously
shown to colocalize in Glut4 vesicles and translocate to the plasma membrane (Uphues et al.,
1994), did not translocate in response to insulin and that rab4A level in the plasma membrane
was enhanced in the basal state (Uphues et al., 1995). Thus it was suggested that in the fatty
rat heart, Glut4 was being redistributed from the intracellular store to the cell surface in the
basal state. However, it was shown in the present study as well as by Uphues et al. (1995)
that there was a significant increase in Glut4 in the plasma membrane in response to insulin
without changing the Glut4 content in the intracellular membrane. This may be due to the translocation of a second pool of Glut4 which may become activated with maximal insulin stimulation in the fatty rat heart. Two separate pools of intracellular Glut4 has been proposed in skeletal muscle due to observations in biochemical studies that the effect of insulin and contraction on glucose transport (Nesher et al., 1985; Zorzano et al., 1986; Wallberg-Henriksson et al., 1988) and on Glut4 translocation (Gao et al., 1994) was additive. However, the additive effect of these two stimuli on glucose transport and Glut4 translocation was not supported by others (Douen et al., 1990; Goodyear et al., 1990; Brozinick et al., 1994). The controversial results reported from these studies may be due to differences in the subcellular fractionation technique which could have caused different degrees of contamination in membrane fractions. Very recently, Lund et al. (1995) used a photolabeling technique to quantitate cell surface Glut4 to assess translocation in response to insulin and contraction. It was shown that maximal stimulation with insulin and contraction produced effects greater than either stimulus alone. In addition, the degree of Glut4 recruitment to the plasma membrane correlated closely with the stimulation of glucose transport (Lund et al., 1995). More importantly, it was shown that inhibition of phosphatidylinositol 3-kinase using wortmannin, abolished the effects on glucose transport and Glut4 translocation caused by insulin but not by contraction (Lund et al., 1995). This provided evidence that contraction stimulates Glut4 translocation through a mechanism distinct from that of insulin. Another line of evidence supporting the existence of two separate pools of Glut4 in skeletal muscle is provided by the finding that contraction caused Glut4 recruitment to the plasma membrane while Glut4 content in the insulin-regulated pool remained the same (Douen et al., 1990; Etgen et al., 1993). Glut4 recruitment to the plasma membrane in response to contraction and insulin was associated with a similar increase in total GTP-binding proteins in the plasma membrane (Etgen et al., 1993). Total GTP-binding proteins in the insulin-regulated compartment decreased in response to insulin but not to contraction. It was also reported that detailed analysis of the GTP-binding proteins in the plasma membrane fraction showed
differences in the magnitude of increase in two of the GTP-binding proteins (25-kDa and 29-kDa) due to the two different stimuli (Etgen et al., 1993). This may suggest the involvement of a different GTP-binding protein in mediating contraction-stimulated Glut4 mobilization in skeletal muscle. So far there is only one report on the effect of contraction in the heart. Kolter et al. (1992) have demonstrated that electrical stimulation of isolated cardiac myocytes induced glucose transport and Glut4 translocation with an effect similar to that of insulin. It was also shown that stimulation with both insulin and contraction did not produce effects greater than either stimulus alone (Kolter et al., 1992). However, electrical stimulation with a frequency of only 5 Hz was used in this study. It has been shown that such a frequency produced only half maximal effects in skeletal muscle (Lund et al., 1995). Further experiments would be required to confirm whether the effects of contraction and insulin are additive in the heart. Although at present there is no evidence from biochemical studies that two separate pools of intracellular Glut4 exist in cardiac tissue, results from immunocytochemical studies have indicated that Glut4 is distributed in several locations inside the cell (Slot et al., 1991b). It is not known whether these distinct cellular locations of Glut4 represent separate functional pools of Glut4 in the cardiac cell.

VII) Future Experiments

The present study has demonstrated a lower Glut4 content in the plasma membrane and intracellular membrane of the fatty Zucker rat heart. Nevertheless, Glut4 recruitment to the plasma membrane was shown to be similarly responsive to insulin although the absolute level of Glut4 was reduced both in the basal and insulin-stimulated state. Since it has been previously shown that vanadium treatment in the fatty Zucker rat could markedly enhance glucose utilization in the heart without changing the expression of Glut4 (Brichard et al., 1992), it may be possible that vanadium mediates its effects in enhancing Glut4 translocation. Using the methods of subcellular fractionation and competitive ELISA in the present study, it
was demonstrated that cardiac Glut4 translocation \textit{in vivo} can be assessed. This method will allow for the future study of cardiac Glut4 translocation in fatty Zucker rats treated with vanadium. From the results obtained in this study, the ability of vanadium treatment to enhance maximal cardiac Glut4 translocation can be assessed by using insulin doses from 2.5 U/kg to 10 U/kg and with a stimulation period of 5 to 30 minutes.
CONCLUSIONS

1. In the fatty Zucker rat, the apparent reduction in plasma membrane and intracellular membrane Glut4 content may suggest a lower cardiac expression of Glut4. However, the reduction in Glut4 content was associated with a similar decrease in Na\(^+\)/K\(^+\) ATPase activity. Further experiments are required to confirm these findings.

2. The dose response curve for cardiac Glut4 translocation \textit{in vivo} showed that a maximal response was already reached at an insulin dose of 2.5 U/kg and there was no further response at higher insulin doses of 5 and 10 U/kg.

3. The time profile of cardiac Glut4 translocation \textit{in vivo} showed that maximal translocation was already reached at 5 minutes after the injection of insulin and persisted for another 25 minutes.

4. In fatty Zucker rats, Glut4 recruitment to the plasma membrane was responsive to insulin with a magnitude similar to that in lean Zucker rats. However, Glut4 recruitment to the plasma membrane was not associated with a decrease in Glut4 content in the intracellular membrane fraction in the fatty rat heart. These results are not understood at present and further experiments are required to confirm this finding.
REFERENCES


Smith RM, Charron MJ, Shah N, Lodish HF, Jarett L. Immunoelectron microscopic demonstration of insulin-stimulated translocation of glucose transporters to the plasma


Takizawa PA, Malhotra V. Coatomers and SNAREs in promoting membrane traffic. Cell 75: 593-596, 1993.


Walhe KWJ, Milne L, McIntosh G. Regulation of polyunsaturated fatty acid metabolism in tissue phospholipids of obese (fa/fa) and lean (Fa/-) Zucker rats. 1. Effect of dietary lipids on cardiac tissue. Lipids 26: 16-22, 1991.


