MYOCARDIAL GLUCOSE TRANSPORT SYSTEM RELATED PROTEINS: THE EFFECTS OF AGING AND EXERCISE IN OLD MICE

by

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B.Sc. Concordia University, 1994

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of
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Degree: Master of Science

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ABSTRACT

This thesis comprises two related studies:

"The effects of age on myocardial glucose transport system related proteins of the mouse." This study examined the effects of aging on the myocardial protein content of Glut 4, Glut 1, insulin receptor, IGF-1 receptor, and PI3 kinase of the C57Bl/6 mouse. Insulin and IGF-1 receptor autophosphorylation activity was also assessed. Glut 4 content was increased fourfold between 22.5 and 29 mo. of age, as compared to 5 mo. animals. Glut 1 content was increased by 40% at 17 mo., returning to 5 mo. levels at 29 mo. Insulin and IGF-1 receptor content decreased early between 5 and 17 mo. by 20 to 35%, and remained constant until 29 mo. and 25 mo. respectively. Between 25 and 29 mo., IGF-1 protein levels decreased to 24% of 5 mo. control. Receptor autophosphorylation was increased at basal and maximally stimulating (10^{-6} M) concentrations of insulin or IGF-1 by two to fourfold. PI3 kinase content decreased 30% by 12 mo. and remained constant thereafter. We conclude that the components of the myocardial glucose transport system studied here are upregulated with advancing age in accordance to the increased importance of cardiac glucose metabolism with aging.

"The effects of exercise on glucose transport system related proteins of the aged mouse myocardium." The effects of 9 months of chronic voluntary exercise, 2.5 hours of forced acute exercise, and a combination of both treatments, on content of myocardial glucose transport system related proteins were assessed in 24 month old C57Bl/6 mice. Specifically, ventricular content of Glut 4 and Glut 1 transporters, insulin and IGF-1 receptors, and PI3 kinase was quantified by immunoblot. Chronic exercise was ineffective at altering the contents of any proteins, but potentiated the effects of acute

exercise. Acute exercise in combination with chronic exercise caused increases of 114% in Glut 1 content and 33% in PI3 kinase content, expressed per mg wet tissue. Acute exercise alone or in combination with chronic exercise decreased insulin receptor content by 34% and 44% respectively. We conclude that the aged mouse myocardial glucose transport system retains the capacity to respond to exercise.

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TABLE OF CONTENTS

Approval ii
Abstract iii
Acknowledgements v
Table of Contents vi
List of tables and figures ix

Chapter 1: Introduction 1
1.1 Review of the literature 1
1.1.1 Background 1
1.1.2 The muscle glucose transport system in detail 8
1.1.2.1 The glucose transporters 8
1.1.2.2 The insulin and the IGF-1 receptors 13
1.1.2.3 Signalling intermediates 16
1.1.2.4 Other mechanisms 17
1.1.3 The effects of aging and exercise on the aged muscle glucose transport system 19
1.1.3.1 Aging 19
1.1.3.2 Exercise and the aged muscle glucose transport system 22
1.2 Purpose and Hypotheses 26
1.3 References 28
1.4 Figures 39

Chapter 2: The effects of age on myocardial glucose transport system related proteins of the mouse 41
Abstract 42
2.1 Introduction 43
2.2 Methods 45

2.3 Results.......... 48
2.4 Discussion.......... 51
2.5 Acknowledgements.......... 56
2.6 References.......... 56
2.7 Tables and figures.......... 59

Chapter 3: The effects of exercise on glucose transport system related proteins of the aged mouse myocardium.......... 72
Abstract.......... 73
3.1 Introduction.......... 74
3.2 Methods.......... 76
3.3 Results.......... 79
3.4 Discussion.......... 80
3.5 Acknowledgements.......... 85
3.6 References.......... 85
3.7 Tables and figures.......... 89

Chapter 4: Integrated conclusion and future directions
4.1 Summary and conclusions.......... 95
4.2 Future directions.......... 96
4.3 References.......... 98

Appendices:
Appendix A: Aging study raw data.......... 100
Summary table.......... 101
Summary figures.......... 102
Raw data spreadsheets.......... 104
Photos of analyzed western blot films.......... 111
Appendix B: Exercise study raw data.......... 116

Summary table ... 117
Summary figures ... 118
Raw data spreadsheets ... 120
Photos of analyzed western blot films ... 125
Appendix C: Detailed Methods ... 130
Western blot protocol ... 131
Tissue preparation ... 133
Electrophoresis ... 137
Electrotransfer ... 142
Immunodetection ... 144
Densitometry ... 148
Receptor autophosphorylation and substrate phosphorylation assays ... 152
Receptor radioligand binding assay protocol ... 159
Immunoprecipitation procedure ... 162

LIST OF TABLES AND FIGURES

Chapter 1

Figure 1.1 Schematic representation of the muscle glucose transport system 40

Chapter 2

Table 2.1 Descriptive data for age groups 60

Table 2.2 Summary of western blot results 61

Table 2.3 Summary of receptor autophosphorylation results 62

Table 2.4 Correlation table 63

Figure 2.1 Graphical summary of western blot results expressed per 200 µg protein 64

Figure 2.2 Graphical summary of western blot results expressed per mg wet tissue 65

Figure 2.3 Graphical representation of autophosphorylation assay results 66

Figure 2.4 Graphical representation of samples over-expressing Glut 4 67

Figure 2.5 Scatter diagrams for significant correlations 68

Figure 2.6 Graphical comparison of variable ratios across age groups 69

Figure 2.7 Representative immunoblots 70

Figure 2.8 Schematic representation of the muscle glucose transport system 71

Chapter 3

Table 3.1 Descriptive data for exercise groups 90

Table 3.2 Summary of western blot results 91

Figure 3.1 Graphical summary of western blot results expressed per 200 µg protein 92

Figure 3.2 Graphical summary of western blot results expressed per mg wet tissue 93

Figure 3.3 Representative immunoblots 94

Chapter 1

INTRODUCTION

1.1 Review of the literature

1.1.1 Background

Human aging is associated with a decrease in the ability of insulin to stimulate glucose uptake [23]. A state of glucose intolerance, defined as increased time of disposal of an oral or intravenous bolus of glucose, often develops with advancing age [25][54]. Studies using the euglycemic clamp technique [34][86][88] and the intravenous glucose tolerance minimal model [20] have demonstrated that this age-related impairment in glucose tolerance is due in large part to development of peripheral tissue insulin resistance [54]. A delay in glucose-induced suppression of hepatic glucose output also contributes to age-related glucose intolerance [54].

Since skeletal muscle is the major site of insulin-mediated glucose disposal [54], a decline in glucose clearance in older subjects appears to be due to skeletal muscle insulin resistance [25]. This insulin resistance is hypothesized to be the result of a yet to be discovered defect in the muscle glucose transport system [47]. This system encompasses many proteins, namely signal transducer proteins and transport proteins, all operating under tight regulation. In the intact system, circulating insulin released by the pancreas binds to muscle cell surface receptors, initiating a cascade of biochemical events which cause the translocation of glucose transporters from an intracellular storage site to the muscle plasma membrane, thereby enhancing glucose uptake into the cell. For a review see [7]. The rate of insulin-stimulated glucose uptake is largely determined by the number of these insulin-responsive transporters in the membrane [108]. Many studies have attempted to elucidate which component of this system could be

responsible for the deterioration of insulin-mediated transport. There is some evidence for decreased transporter content [42][5], as well as decreased receptor content and activity [5][22] but the findings are not conclusive. Ongoing research efforts are focussing on the characterization of these and other insulin signal transduction elements which are candidates for a localization of a defect. However, all the details of the signalling cascade still remain to be elucidated.

It is believed that muscle insulin resistance is the primary defect in a chain of events which can lead to non-insulin-dependent diabetes mellitus (NIDDM), and is in fact considered a state of pre-diabetes [47]. Defective signal transduction of insulin at the target tissue requires hypersecretion of insulin by the pancreas in order to maintain euglycemia. As a result, a state of basal and post-prandial hyperinsulemia develops [47]. If the insulin resistance is severe enough, the high levels of circulating insulin are insufficient to stimulate proper glucose disposal and hyperglycemia develops [47]. Hyperinsulemia together with hyperglycemia greater than 7.8 mM are characteristics of NIDDM [9].

Insulin resistance is associated with, and is thought to be the initial defect leading to, a host of anomalies including increased levels of very low density lipoprotein (VLDL), hypertriglyceridemia, decreased high density lipoprotein (HDL) cholesterol, and hypertension [9]. Together these defects make up Metabolic Syndrome of western society, and along with obesity and NIDDM, are all considered risk factors for the development of atherosclerosis and coronary heart disease [6].

Another hormone known to stimulate glucose uptake in muscle is insulin-like growth factor 1 (IGF-1). This hormone acts through its own cell-

surface receptor to stimulate glucose transporter translocation by a mechanism similar to, and probably converging with, that of insulin [24]. The role of IGF-1 in muscle glucose transport has received less attention than insulin because it is less potent in stimulating glucose transport [64] due in part to a fourfold lower IGF-1 receptor content as compared to insulin receptor content [22]. It has been suggested that IGF-1 could be used to stimulate glucose transport in insulin-resistant states such as diabetes and the use of IGF-1 as a potential therapeutic tool is presently under investigation. Experiments in humans with extreme insulin resistance syndromes caused by mutated insulin receptors have demonstrated a beneficial blood glucose lowering effect of IGF-1 [62][93]. However there is some evidence that age-related IGF-1 resistance may occur in parallel with insulin resistance [22]. This would imply that the post receptor defect is located in a signalling step common to both the insulin and the IGF-1 mechanisms, but this remains to be demonstrated experimentally.

Like skeletal muscle, cardiac muscle is also insulin-sensitive tissue. Cardiac muscle is a highly oxidative tissue and in the aerobically perfused heart, free fatty acid catabolism provides the major source of energy for adenosine triphosphate (ATP) resynthesis, accounting for between 60 and 70% under basal conditions, with glucose and lactate catabolism providing the rest [104]. However, the contribution of free fatty acid oxidation to overall cardiac metabolism decreases with increasing age [1][46] and thus the importance of glucose oxidation and glycogenolysis increases in terms of energy provision [66].

While of quantitatively lesser importance than skeletal muscle for glucose disposal, age-related insulin resistance of the heart is nevertheless a serious concern in different pathological conditions during which glucose can

become a more important metabolic fuel than fatty acids: 1) In responding to acute energy deficit, the working heart cannot rapidly increase uptake and metabolism of free fatty acids and relies on increased glycolysis and glucose oxidation brought about by accelerated glucose transport and glycogenolysis [37]; 2) Similarly, in conditions of chronic increase in workload, such as hypertension and the associated concentric hypertrophy, there is an increase in glucose uptake and a corresponding decrease in free fatty acid utilization [101][2]; 3) In the extreme case of myocardial ischemia, the heart must rely primarily on anaerobic glycolysis of exogenous glucose and endogenous glycogen as oxygen deprivation renders the myocardium incapable of fatty acid β oxidation and oxidative phosphorylation [74]. During severe ischemia, cardiac glycogen stores can be depleted within 1-2 hours [75] and thus any process that limits exogenous glucose availability, such as a compromised glucose transport system, can adversely affect myocardial function and survival.

Deficiency of oxygen at the tissue level, as occurs in hypoxia and ischemia, has been shown to trigger glucose transporter translocation to the cell membrane and increase glucose uptake [100]. The exact mechanism by which the signal is transduced is unknown, but is thought to be distinct and additive to insulin mechanism [100]. Recruitment of glucose transporters to the plasma membrane increases the ability of the myocyte to take up glucose and metabolize it anaerobically when the oxygen supply is limited. During an ischemic episode, administration of insulin has been shown to improve cardiac function and result in less extensive myocardial injury [100]. The cardioprotective effect of insulin is the result of a translocation of more transporters to the cell membrane than are recruited by ischemia alone. A decrease in the insulin sensitivity of the myocardium would obviously

reduce the effectiveness of this form of pharmacological intervention. It is unknown whether IGF-1 could be used in the place of insulin.

There is evidence that during diabetes, cardiac muscle exhibits insulin resistance similar to that observed in skeletal muscle [37]. Diabetes is thought to impact on the functioning of the myocardial glucose transport system as diabetic patients demonstrate decreased myocardial glucose uptake [4]. Furthermore, diabetic patients presenting with acute myocardial infarction experience poorer survival and increased frequency of myocardial rupture and congestive heart failure [97]. Diabetes has been shown to be associated with a significant decrease in myocardial insulin-regulatable glucose transporter content [37][45].

Less is known, however, about whether the myocardial glucose transport system experiences age-related changes and develops insulin resistance similar to its skeletal muscle counterpart. Very few studies have attempted to document changes in various components of the myocardial glucose transport system during aging. Only insulin-regulatable transporter content has been quantified with aging, and this only by one group [15][44]. A small decrease was reported, which would appear counter-intuitive in light of the increased contribution of glucose metabolism to overall cardiac metabolism with aging [66]. If the myocardial system does behave like the skeletal muscle system, then the heart may become compromised in the face of a metabolic challenge described above.

One of the purposes of the studies presented in this thesis is to quantify age-related changes in several components of the myocardial glucose transport system in an attempt to document whether this system may be adversely affected by the normal aging process.

It appears that while aging may cause detrimental changes to the glucose transport system and insulin resistance, lifestyle may have a large impact on whether these changes lead to more serious metabolic derangements. It is believed that the high fat and refined sugar diet, physical inactivity and stress, all common to highly industrialized countries, contribute to the prevalence of Metabolic syndrome and NIDDM in the Western world [6]. The postulated mechanisms by which these three factors act are complex and multi-faceted, but only physical activity has a direct effect on the glucose transport system. Muscular contraction has been shown to cause recruitment of glucose transporters to the muscle plasma membrane and therefore increase glucose uptake [50][29][38]. Of special relevance to the work presented in this thesis are the observations that this effect of contraction appears to be transduced by way of a signalling mechanism distinct from, and additive to, that of insulin [36] and appears to remain normal in insulin-resistant muscle [14].

For these reasons, physical exercise is an effective way to lower blood glucose in the presence of decreased insulin sensitivity and is now recognized as an important tool for the management of diabetes [9]. It has been proposed that exercise can help prevent hyperglycemia and can thus halt the progression of pre-diabetic insulin resistance to full blown diabetes [9]. This has been supported by epidemiological studies suggesting that exercise reduces the risk for developing NIDDM [102][48][65].

Exercise has been shown to enhance the skeletal muscle glucose transport system. Insulin sensitivity is increased by both acute and chronic exercise, and this effect is persistent for up to 2 days after a bout of acute exercise and up to 4 days following the last bout of chronic exercise [10]. Enhanced insulin-stimulated glucose transport has also been demonstrated in

aged muscle [17]. Chronic activity has been shown to increase insulin-regulatable transporter content in a variety of skeletal muscle [84][83], while inactivity has been shown to decrease it [68]. Increase in glucose transporter content of aged skeletal muscle also appears to be possible with exercise training [109]. Other components of the skeletal muscle glucose transport system have received less attention, but insulin receptor number does appear to increase with exercise training [28][90].

Exercise also affects the myocardial glucose transport system. It has been shown that exercise training increases cardiac glucose uptake during rest and exercise [56], increases glycogen stores [92], and is cardioprotective against hypoxia [105]. It is well documented that the aged myocardium responds positively to exercise training, as shown by improved myocardial function and aerobic energy metabolism [98]. Also, exercise training has been shown to attenuate the decrease in myocardial glucose transporter content observed in experimentally-induced diabetes [45]. This may explain how exercise training also partially normalizes myocardial glucose utilization depressed by diabetes [76]. However, it has been reported that exercise training was ineffective at attenuating the small age-related decrease in myocardial transporter content [44]. It is unknown if exercise can upregulate the content or function of other components of the myocardial glucose transport system.

The second purpose of the studies presented in this thesis is to investigate the effects of exercise on several components of the aged myocardial glucose transport system heretofore not studied.

1.1.2 The muscle glucose transport system in detail

The muscle glucose transport system will refer to striated muscle only, i.e. skeletal muscle and cardiac muscle. The system is composed of the glucose transporters Glut 1 and Glut 4, the Glut 4 trafficking system, the membrane receptors for insulin and IGF-1, and the various components of the intracellular signalling cascade responsible for signal transduction and stimulation of Glut 4 translocation. Very little is known about the trafficking system, and similarly, only the first few proximal events of the signalling cascade have been identified. Due to the enormous complexity of the muscle glucose transport system, the determination of all physiological and pathophysiological sites of regulation within this system is an ongoing challenge. See figure 1.1 for a schematic representation of the main components of the muscle glucose transport system and their interactions.

1.1.2.1 The glucose transporters

Glucose is a hydrophillic molecule requiring carrier mediated transport for cellular uptake. Mammalian glucose transporters are classified as either facilitative transporters or co-transporters (69). The latter class includes Sglt 1, the Na^+ dependent glucose co-transporter found in the intestinal brush border and in the renal tubular epithelium (95). This transporter arises from a different gene family than the facilitative glucose transporters (69) and is not relevant to the work presented here.

To date, six mammalian facilitative glucose transporter genes have been identified. For reviews see (95) and (69). Their protein products are designated Glut 1 to 5 and Glut 7. The amino acid sequences of these isoforms have all been deduced from cloned cDNA. The mammalian GLUT genes, located on different chromosomes, belong to a large superfamily of genes

whose protein products are involved in the transport of a variety of hexoses and other carbon compounds. The characteristic feature of these proteins is the presence of 12 transmembrane segments, as predicted by hydropathy plots [69]. Human Glut 1 to 5 exhibit 39 to 65% amino acid sequence identity and 50 to 76% sequence similarity in pairwise comparisons [8]. The six transporter isoforms exhibit a tissue specific distribution and a single cell type often expresses two or more different isoforms. The expression of most of the GLUT genes is developmentally regulated and, in the adult tissue, at least three of the genes are known to be subject to regulation by a number of endogenous factors [69].

Glut 1 often called the erythrocyte or brain glucose transporter, is found in many fetal and adult tissues including red blood cells, endothelia, and striated muscle. It is a 55 kDa glycoprotein whose proposed function in muscle involves basal glucose transport. It is the most widely studied of the transporters in terms of its structure/function and its kinetics, due to its very abundant expression in erythrocytes [95]. As early as 1952, a saturable carrier mechanism exhibiting simplified Michaelis-Menton kinetics was proposed to predict glucose transport in red blood cells. Controversy exists to this day as to whether any type of simple carrier mechanism can explain the complex kinetics of Glut 1 glucose transport. Recent evidence suggesting that these transporters form oligomers in membranes obviously lends support to this view [95]. The most widely accepted mechanism is the alternating conformation model which states that loaded or unloaded substrate binding sites are alternatively exposed at one face of the membrane or the other as the protein undergoes conformational changes. It is suggested that amphipathic helices cluster together to form the walls of an aqueous pore across the lipid bilayer [95].

Glut 2 is the major glucose transporter isoform expressed in hepatocytes, pancreatic β cells and absorptive epithelial cells of intestinal mucosa and kidney [69].

Glut 3 is the most prominent isoform expressed in parenchymal cells of the adult brain. In the mouse, its expression is limited to nervous tissue, whereas in human, the protein has been detected in a wide variety of tissues including placenta, liver and kidney [69].

Glut 4, a 45 kDa glycoprotein also called the insulin-regulatable glucose transporter, is expressed only in adipocytes and muscle cells [69]. These are considered insulin-sensitive cell types as they respond to insulin with a rapid and reversible increase in glucose transport mediated by Glut 4 [69]. This transporter will be discussed in more detail below.

Glut 5, believed to be a fructose transporter, has been identified in the apical membrane of intestinal enterocytes, in the plasma membrane of mature spermatozoa, as well as in brain endothelium [69].

Glut 7 is the most recently discovered isoform and has thus far only been detected in the liver. It is believed to be involved in the diffusion of free glucose out of the endoplasmic reticulum of gluconeogenic tissue after the action of microsomal glucose-6-phosphatase on glucose-6-phosphate [69].

Of the six mammalian facilitative glucose transporters, only Glut 1 and Glut 4 are involved in skeletal and cardiac muscle glucose transport. Glut 1 is constitutively expressed in the plasma membrane and is believed to mediate basal glucose transport as virtually no Glut 4 can be found in the sarcolemma in the basal state [85]. Glut 1 is expressed in low levels in skeletal muscle and

in significantly higher levels in cardiac muscle [61]. Glut 4 is the predominant isoform in striated muscle. Glut 4 content is muscle type specific and positively correlated to muscle oxidative capacity. Thus Glut 4 is most abundant in myocardium [99] and progressively less abundant in red and white skeletal muscle [67][39]. Maximal rate of muscle glucose transport is highly correlated to Glut 4 content in a variety of muscles [61][96]. However, change in Glut 4 content does not always reflect a change in transport capacity [109] as Glut 4 trafficking is also an important site of regulation.

In a basal state, most Glut 4 molecules are located in secretory tubulo-vesicular structures [85]. Upon stimulation by insulin, as well as by IGF-I, muscular contraction or hypoxia, a fraction of total cellular Glut 4 is translocated to the sarcolemma [16]. It has been reported that the T-tubule may be a more exact site of translocation of Glut 4 and, thus it has been suggested that glucose transport may occur predominantly across the T-tubule membrane under basal and stimulated states [30].

The translocation process of Glut 4 from intracellular pools to the plasma membrane appears to be achieved through an exocytosis-like mechanism which has many similarities to secretory phenomena such as fusion of neurotransmitter vesicles at synapses and hormone release from endocrine cells. Similarly, recycling to intracellular pools is thought to occur by an endocytosis mechanism. Recycling is believed to occur continuously during stimulated translocation. A current model for trafficking of Glut 4 [51] suggests that, in the basal state, most transporters are located in specialized tubulo-vesicular compartments analogous to secretory compartments. A small percentage of transporters are also located in early endosomes. The tubulo-vesicular pool provides a reservoir of transporters for rapid exocytosis to the plasma membrane in response to stimulation. Processing of the

transporters in the membrane is thought to occur through occluded and partially occluded vesicles, which act as a precursor state to complete integration into the plasma membrane. Glut 4 recycling occurs through clathrin-coated pits which are endocytosed into an early endosome pool and then sequestered and sorted to reform the specialized tubulo-vesicular compartment. This model is supported by detection of Glut 4 in these various subcellular locations by photolabelling, subcellular fractionation, and immunocytochemical studies. For a review see [(51)].

The multiple subcellular locations in which Glut 4 has been identified and the limitations of the techniques used to isolate Glut 4 pools may be at the root of many controversies surrounding Glut 4 trafficking and glucose transport function (see below and section 1.1.2.4). Western blots of plasma membrane fractions can detect Glut 4 in all plasma membrane forms [(51)]. However, transporters in occluded vesicles and semi-occluded vesicles, which are not fully exposed at the cell surface and which may be associated with trafficking proteins, may be omitted during quantitation by photolabelling or binding studies [(51)]. Also, subcellular fractionation techniques are unable to isolate all the various subcellular pools of Glut 4 transporters, and thus necessarily combine some of these pools.

One of the controversies surrounding Glut 4 involves its transport kinetics. It has been suggested that Glut 4 regulation may involve not only translocation, but also transporter intrinsic activity. This line of thinking arises from findings that increases in the fraction of plasma membrane transporter cannot always account for the observed increases in glucose uptake [(50)(71)(49)(12)]. These studies also demonstrate that binding affinity of the transporter does not appear to change. Thus it has been proposed that transporter Vmax may be regulatable but not its Km. This model of two-site

regulation is still quite popular today, despite new techniques such as photolabelling which have been used to demonstrate that insulin-stimulated glucose transport can be accounted for completely by translocation of Glut 4 transporters to the plasma membrane [108][82]. Furthermore, kinetic studies of glucose transport by Glut 1 clearly show that glucose transport kinetics cannot be explained by simple first-order enzyme kinetics.

1.1.2.2 The insulin and the IGF-1 receptors

Insulin and IGF-1 are both part of a family of structurally and evolutionarily related peptide hormones. Insulin and IGF-1 share 49% amino acid sequence identity [24].

Insulin is secreted from pancreatic β cells in response to elevated blood glucose concentrations. Its metabolic and mitogenic effects are numerous, but only its role in acutely increasing muscle glucose uptake will be discussed in this review. Its effects on muscle are mediated via a high specificity cell surface receptor, the insulin receptor. This receptor is necessary to transduce the chemical signal of insulin in the bloodstream into intracellular events which ultimately induce the translocation of Glut 4 from intracellular pools to the muscle plasma membrane [21]. The post-receptor signalling cascade is discussed in 1.1.2.3.

IGF-1 is released by the liver and secreted locally by various tissues including skeletal muscle, and is capable of acting in an autocrine, a paracrine or an endocrine manner [91]. Like insulin, IGF-1 has many mitogenic effects as well as metabolic effects, and like insulin, IGF-1 stimulates the translocation of Glut 4 to the sarcolemma, thereby increasing glucose transport into the muscle cell [64]. IGF-1 acts through its own high specificity membrane receptor, the IGF-1 receptor [64]. However, it is believed that the metabolic

signalling cascade activated by IGF-1 is the same as that activated by insulin [24].

Like their ligands, the insulin and the IGF-1 receptors are structurally related. Muscle contains abundant amounts of both insulin and IGF-I receptors [22]. Cross-reactivity studies have demonstrated the specificity of these receptors for their specific ligand. The affinity of the insulin receptor for insulin is two orders of magnitude greater than for IGF-I [64]. Similarly, insulin has a minimal effect on IGF-I binding to the IGF-I receptor [64]. IGF-1 is believed to be less potent than insulin for stimulating glucose transport [64], perhaps due to a fourfold lower IGF-1 receptor content in adult skeletal muscle, as compared to insulin receptor content [22].

The structure and function of these receptors are reviewed in [55] and [24]. They are allosteric enzymes with a regulatory protein subunit, the α-subunit, and a catalytic subunit, the β-subunit [55]. The α-subunit for each receptor contains the ligand binding domain and is entirely extracellular. The β-subunit contains a single transmembrane domain so that approximately one third is extracellular and two third intracellular. The β-subunit contains a tyrosine specific protein kinase domain, whose activity is suppressed by the α-subunit. A receptor is organized as a functional dimer with an $\alpha_2 \beta_2$ structure, stabilized by a small number of α-α and α-β disulfide bridges [24].

The binding of ligand molecules to the receptor's α-subunits removes the inhibition of this subunit on the β-subunit's intrinsic activity by a hypothesized propagated conformational change [55]. This inhibition has also been removed experimentally by mutagenesis and tryptic cleavage [55]. This conformational change activates a tyrosine specific protein kinase catalytic domain of the β-subunits causing phosphorylation of at least five tyrosine

residues on the opposite β-subunit of the dimer in a transphosphorylation mechanism. Autophosphorylation of the β-subunits in this way is autocatalytic and activates the receptor's tyrosine kinase activity toward other endogenous substrates [55], discussed in 1.1.2.3 below. It is believed that activated insulin and IGF-1 receptors phosphorylate the same endogenous substrate and thus use the same signalling cascade to induce translocation of Glut 4 transporters. A converging pathway may explain findings suggesting that age-related IGF-1 resistance occurs in parallel with insulin resistance [22].

The receptors are organized as functional dimers, and thus, each receptor contains two α-subunits and two ligand binding sites. Allosteric interaction between the binding sites results in non-classical ligand-receptor binding kinetics characterized by negative cooperativity [24]. The insulin and the IGF-1 receptors bind only one ligand molecule with high affinity and the second ligand molecule with about tenfold lower affinity, as calculated by Scatchard analysis of binding assays. This is thought to be physiologically important for the regulation of the dissociation rate of the ligand as a function of ambient ligand concentration [24].

On binding of their ligand, the receptors aggregate along the plasma membrane and are rapidly internalized. Following internalization, the receptors may be degraded or recycled to the cell surface [55].

There is evidence for the existence of insulin/IGF-1 receptor hybrids composed of an insulin receptor αβ-heterodimer and an IGF-1 receptor αβ-heterodimer [94]. The function of these hybrids has been shown to be more akin to that of the IGF-1 receptor than that of the insulin receptor [73]. The physiological importance of these hybrids is unknown.

1.1.2.3 Signalling intermediates

Remarkable progress has been made over recent years in elucidating the cascade of biochemical signalling events triggered by insulin and IGF-1 to promote their metabolic and mitogenic effects. Most notably, the tyrosine kinase activity of the insulin receptor and IGF-1 receptor has been linked with the activation of a network of interacting proteins, which in turn activate a number of downstream signalling systems. These systems and the interactions between them are highly complex and thus only events related to Glut 4 translocation to the muscle plasma membrane will be discussed in this review. The following is based on a 1995 review by Czech [21]. Refer to [106][24][33] for additional reviews.

It is now known that the most proximal event in the signalling cascade initiated by insulin or IGF-1 is the tyrosine phosphorylation of the endogenous cellular protein IRS-1, or insulin receptor substrate 1, by the β subunit of the insulin and IGF-1 receptors. IRS-1 is a 131 kDa protein with multiple tyrosine and serine phosphorylation sites which lacks any known catalytic activity [21]. The apparent function of the IRS-1 tyrosine phosphates is to act as docking sites, recruiting specific proteins that bind these sites via specialized domains denoted SH2, or Src homology 2. Two proteins are known to associate with the docking sites on IRS-1: phosphatidylinositol 3 kinase, or PI3 kinase, and the tyrosine phosphatase SH-PTP2. The activity of these proteins is significantly stimulated upon binding of their SH2 domains to tyrosine phosphorylated peptides.

Insulin-regulated PI3 kinase is a dimeric protein consisting of a regulatory 85 kDa subunit, p85, and a 110 kDa catalytic subunit, p110, that promotes the phosphorylation of phosphatidylinositol at the D3 position of the inositol ring producing PI-3-P, PI-3,4-P_2 and PI-3,4,5-P_3. Binding of p85 to

p110 is necessary for catalytic activity of the enzyme. Two isoforms, α and β, have been identified for both p85 and p110 and it appears that only PI3 kinase activity associated with p85α is activated by insulin.

It is thought that the accumulation of the above mentioned inositol phosphates in the endosomal membrane system is linked to the trafficking of Glut 4 transporters. However, the exact details remain to be elucidated. In addition, there are probable multiple interactions between the mitogenic signalling cascades activated by the insulin and IGF-1 receptors and the metabolic cascade described above.

It should be noted that glucagon, acting through cAMP, has been reported to impair insulin signalling by decreasing insulin-stimulated receptor and IRS-1 phosphorylation levels, resulting in reduced PI3 kinase / IRS-1 association [(89)]. In addition, activation of protein kinase C (PKC) has been shown to interfere with insulin receptor signalling by inhibiting phosphorylation of IRS-1 and subsequent binding to PI3 kinase [(87)]. These findings would suggest a possible explanation for the decreased insulin-stimulated glucose transport observed in the presence of elevated intracellular calcium concentrations [(81)], as calcium is a known activator of PKC.

1.1.2.4 Other mechanisms

Glut 4 translocation to the plasma membrane is induced by stimuli other than insulin or IGF-1 stimulation. Muscular contraction and hypoxia are both known to increase glucose uptake in striated muscle cells by mechanisms largely unknown.

Studies of cytochalasan B binding to cell-surface glucose transporters have reported that muscular contraction increases Glut 4 content in the plasma membrane [50]. Subcellular fractionation has also been employed to measure Glut 4 content of the plasma membrane and of intracellular membranous elements, and it has been demonstrated that a concomitant redistribution occurs between these two compartments with contraction [35]. These experiments have been repeated and the effects of contraction have been compared to those of insulin. In some studies it was observed that, unlike insulin, the contraction-stimulated increase in sarcolemmal Glut 4 content was not accompanied by a concomitant decrease in intracellular membrane transporter content [29]. From these studies, it has been suggested that there could exist separate intracellular pools of Glut 4 transporters responsive to different stimuli. This hypothesis is very controversial as other studies have demonstrated concomitant redistribution of transporters [38]. Hypoxia and cardiac ischemia have also been shown to cause translocation of Glut 4 to the plasma membrane and increase glucose uptake [18][100].

It is believed that the mechanism of contraction-stimulated translocation operates by a pathway distinct from the insulin-activated signalling cascade presented above, as the effects of muscular contractions on Glut 4 translocation have been found to be additive to the effects of insulin in several studies [18][78][36]. However, controversy exists here too as other studies have failed to show completely additive effects [13].

Controversy also exists as to whether the effects of hypoxia and ischemia are additive to insulin [18][100]. Hypoxia-stimulated translocation is not additive to contraction-stimulated translocation and thus it is believed that these two stimuli activate converging signalling cascades [18]. The means of signal transduction and the details of these cascades are largely unknown. It

should be noted that muscular contraction has been shown to be a more powerful stimulus for Glut 4 translocation than insulin [36].

The above mentioned controversies may be due to the various subcellular locations in which Glut 4 has been identified and the limitations of the techniques used to isolate Glut 4 pools, as described in section 1.1.2.1.

Of special relevance to the work presented in this thesis are the findings that muscular contraction remains an effective stimulus for Glut 4 translocation in insulin-resistant tissue [11][58].

1.1.3 The effects of aging and exercise on the muscle glucose transport system

1.1.3.1 Aging

The natural aging process is associated with insulin resistance in both skeletal and cardiac muscle, as determined by numerous human and animal studies. The exact molecular site of this resistance is still unclear but many potential sites of regulation within the muscle glucose transport system have been studied.

Skeletal muscle appears to undergo age-related decreases in Glut 4 transporter protein content. However, it would appear that in rats these changes occur during maturation and that content remains stable throughout adulthood. A 14-33% decrease in transporter content has been reported in various red and white muscles between 1 and 10 months of age, with no further change between 10 and 25 [42]. It is noteworthy that some muscles studied did not undergo any age-related decrease in Glut 4. These findings have been disputed by others who have shown no change in Glut 4 content of pooled hindlimb muscles between the ages of 2 and 24 [5] These seemingly

contradicting findings could be explained by either the pooling of muscles masking any effect or by changes occurring before 2 months of age. A trend toward decreased skeletal muscle Glut 4 across the lifespan of rats has been reported in a study that compared young adult (6-8 months) to middle-aged (15-17 months) and very old (27-29 months) [57]. A few other studies have reported age-related changes in Glut 4 content of up to 50%, but very young animals were compared to old animals [63][72]. In humans, a significant negative correlation between age and Glut 4 concentration in a needle biopsy of the vastus lateralis has been demonstrated in subjects ranging between 18 to 80 years of age (r=-0.28 in men and r=-0.51 in women) [52].

By contrast, age-related changes in myocardial Glut 4 protein content appear to occur across the lifespan of the rat. Left ventricular Glut 4 content has been reported to decrease by 15% between 3 and 13 months of age and by an additional 12% between 13 and 25 months [15]. This decrease in mature animals was reconfirmed by the same group in a subsequent study showing a 20% drop in myocardial Glut 4 between 15 and 25 months mirrored by an 11% decrease in Glut 4 mRNA over the same time [44]. While these age-related declines are significant, it is doubtful that myocardial Glut 4 protein content ever reaches levels comparable to even the most oxidative skeletal muscle.

The insulin and IGF-1 receptors have also been studied as potential sites of regulation by the aging process. Skeletal muscle insulin receptor protein content has been shown to fall by approximately 30% between the ages of 6-8 months and 18-20 months [22]. These findings have been contradicted by other studies showing no change in insulin receptor content between 2 and 24 months of age [5]. Most studies have employed ligand binding techniques to estimate receptor content. However, receptor content has recently been measured by immunoblot at four time points between 2 and

20 months of age and was found to remain constant [19]. IGF-1 receptor content has been shown to drop dramatically by 80% between 1 and 6-8 months of age, with only a trend towards further decrease between 6-8 months and 20 months of age [22]. Findings from our laboratory concur as we have recently reported a 40% decrease in IGF-1 receptor protein content between 5 and 12 months of age, with a non-significant trend towards a further decrease between 12 and 24 months [107].

Alterations of receptor function have also been studied. Decreased insulin-stimulated autophosphorylation and tyrosine kinase activity of skeletal muscle insulin receptors have been observed between 2 and 24 months of age [5]. The findings of decreased autophosphorylation have been corroborated by a subsequent study which demonstrated a 25% decrease between 5 and 20 months of age. The findings of decreased receptor tyrosine kinase activity have also been corroborated by another study which showed reduced insulin-stimulated activity between 10 and 20 months but unexplainably reported unaltered insulin-stimulated receptor autophosphorylation [60].

The effects of aging on glucose transport system-related signalling proteins have also been studied. Skeletal muscle IRS-1 protein content has been shown to decrease by approximately 60% between 2 and 5 months of age with no further decrease thereafter up to 20 months. PI3 kinase content remained constant between 2 and 20 months. However, insulin-stimulated phosphorylation of IRS-1 was decreased by 92% between the ages of 12 and 20 months and was accompanied by a decrease in insulin-stimulated IRS-1/PI3 kinase association [19]. This decrease in IRS-1 phosphorylation is in agreement with above-mentioned studies showing decreased tyrosine kinase activity.

The effects of aging on skeletal muscle or myocardial Glut 1 have not been reported nor have the effects of aging on myocardial insulin and IGF-1 receptor content and function or myocardial signalling proteins.

1.1.3.2 Exercise and the aged muscle glucose transport system

In muscle, endurance training induces enzymatic adaptations that increase oxidative capacity. It is believed that changes in Glut 4 transporter content are temporally correlated, and probably co-regulated, with these enzymatic changes [96]. These responses to exercise are undoubtedly of physiological importance in as much as glucose transport is believed to be the rate limiting step in glycolysis of exogenous glucose [59] and that there exists a strong correlation between Glut 4 concentration and maximal rate of glucose transport [96]. Thus increases in glucose transporter concentration serve to increase the proportion of exogenous glucose utilized during exercise and to spare muscle glycogen [96].

Increases in skeletal muscle Glut 4 content in response to a variety of chronic exercise protocols have been extensively documented in animals as well as in humans. Increases of 30% and 44% have been reported in young rat skeletal muscle using a swim training protocol [79][96]. Using long-term treadmill running protocols, increases of 26% to 41% have been reported in various red and white skeletal muscle of young and adult animals [96][32][31]. An intensive 8 day protocol has been shown to cause a 70% increase [70]. A voluntary exercise model of wheel running has also been used successfully to elicit increases. Increases in Glut 4 content of up to 50% have been reported in young animals using this model [40][83][43].

In human studies, muscle biopsy analyses have shown that athlete populations have a 21% greater skeletal muscle Glut 4 content, as compared to sedentary populations [3]. A daily endurance training protocol has been reported to cause a 29% increase in Glut 4 protein content after 5 days of exercise and 36% after 31 days [77]. Diagnosed NIDDM subjects have been reported to show increased Glut 4 content after 9 weeks of cycling exercise [27], while older glucose intolerant subjects have been reported to show a 60% increase with 12 weeks of cycling [53].

Studies on the effects of exercise on muscle Glut 1 are less numerous. A 31% increase in skeletal muscle Glut 1 content has been reported after 1 month of aerobic exercise training in humans [77]. The effects of exercise on myocardial Glut 1 have not been reported.

Changes in skeletal muscle insulin receptor content and activity in response to chronic exercise have also been studied. Treadmill running has been reported to increase insulin receptor content, as deduced from a two fold increase in ligand binding without any change in binding affinity [28]. Similar findings of a 2 to 2.5 fold increase in binding with no change in affinity have been reported using a wheel running model as well [90]. However, these findings have been contradicted by reports of no change in insulin receptor binding or binding affinity following treadmill running [41]. Receptor quantitation by immunoblot has not been reported. In humans, insulin receptor binding and kinase activity have been reported unchanged after 10 weeks of cycling [26]. The effects of chronic exercise on skeletal muscle IGF-1 receptors have not been reported.

Acute exercise is also believed to cause changes to the muscle glucose transport system. It has been reported that one 6 hour bout of swimming is

sufficient to elicit a two fold increase in skeletal muscle Glut 4 mRNA and a 50% increase in total Glut 4 protein measured in rat epitrochclearis 16 hours after the exercise. A second bout of exercise 16 hours after the first bout elicits a further increase in Glut 4 protein without any further increase in mRNA [80]. However, it has been reported that insulin receptor binding, autophosphorylation and kinase activity are all unchanged by a 45 minute bout of treadmill running [103].

It is believed that exercise can also affect the glucose transport system of aged muscle. Acute exercise has been shown to stimulate glucose transport in skeletal muscle of 24 month rats to the same level as maximum insulin stimulation [109]. This same study also reported that skeletal muscle Glut 4 content is increased 55% by 8 weeks of treadmill running in 24 month old rats. However, this increase was not accompanied by an increase in maximally insulin-stimulated glucose transport. Previous studies had proven unsuccessful at eliciting an increase in Glut 4 content [43]. This was probably due to the use of a voluntary exercise model which is often preferred for long term exercise studies in old animals but which may provide a less potent stimulus than a forced exercise model [109]. Our laboratory has demonstrated increases in IGF-1 receptor binding capacity and affinity, with a trend towards increased receptor content, in 24 month old rats following one 2.5 hour bout of exercise [107].

The effects of exercise on the glucose transport system of the aged myocardium have also received some attention. It has been reported that 10 weeks of treadmill running cannot attenuate the 20% reduction in myocardial Glut 4 content observed between 15 and 25 months of age [44]. It should be noted that increases in Glut 4 mRNA were elicited by this protocol. These findings remain to be corroborated and it is quite possible that a longer

exercise stimulus may be necessary to elicit a change in the glucose transport system of this highly oxidative tissue.

The effects of exercise on other components of the aged myocardial glucose transport system have not been reported in the literature.

1.2 Purpose and Hypotheses

Two studies are presented in this thesis. While the studies are co-authored, the manuscripts included in the thesis have been written by the candidate.

Study 1: The effects of aging on myocardial glucose transport system related proteins of the mouse

The purpose of this study was to assess the effects of aging on myocardial glucose transport system related proteins. Specifically, the protein contents of the glucose transporters Glut 1 and Glut 4, the insulin and IGF-1 receptors, and the signalling intermediate PI3 kinase were assessed, as well as the function of the above receptors, across the lifespan of the mouse from maturity to senescence.

It was hypothesized that the aging process would cause a downregulation in the expression and function of the myocardial glucose transport related proteins under investigation.

Study 2: The effects of exercise on glucose transport related proteins of the aged mouse myocardium

The purpose of this study was to examine the effects of acute and chronic exercise on myocardial glucose transport system related proteins in old mice. Specifically, the protein contents of the glucose transporters Glut 1 and Glut 4, the insulin and IGF-1 receptors, and the signalling intermediate PI3 kinase were assessed in control, acutely exercised, chronically exercised and chronically and acutely exercised animals.

It was hypothesized that physical exercise, either acute, chronic, or a combination of both, would cause, in old animals, an upregulation in the expression of the myocardial glucose transport system proteins under investigation, as compared to age-matched controls.

These two novel studies were intended to contribute to the understanding of the development of muscle insulin resistance and its devastating sequelae as well as to its treatment and possible prevention.

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1.4 Figures

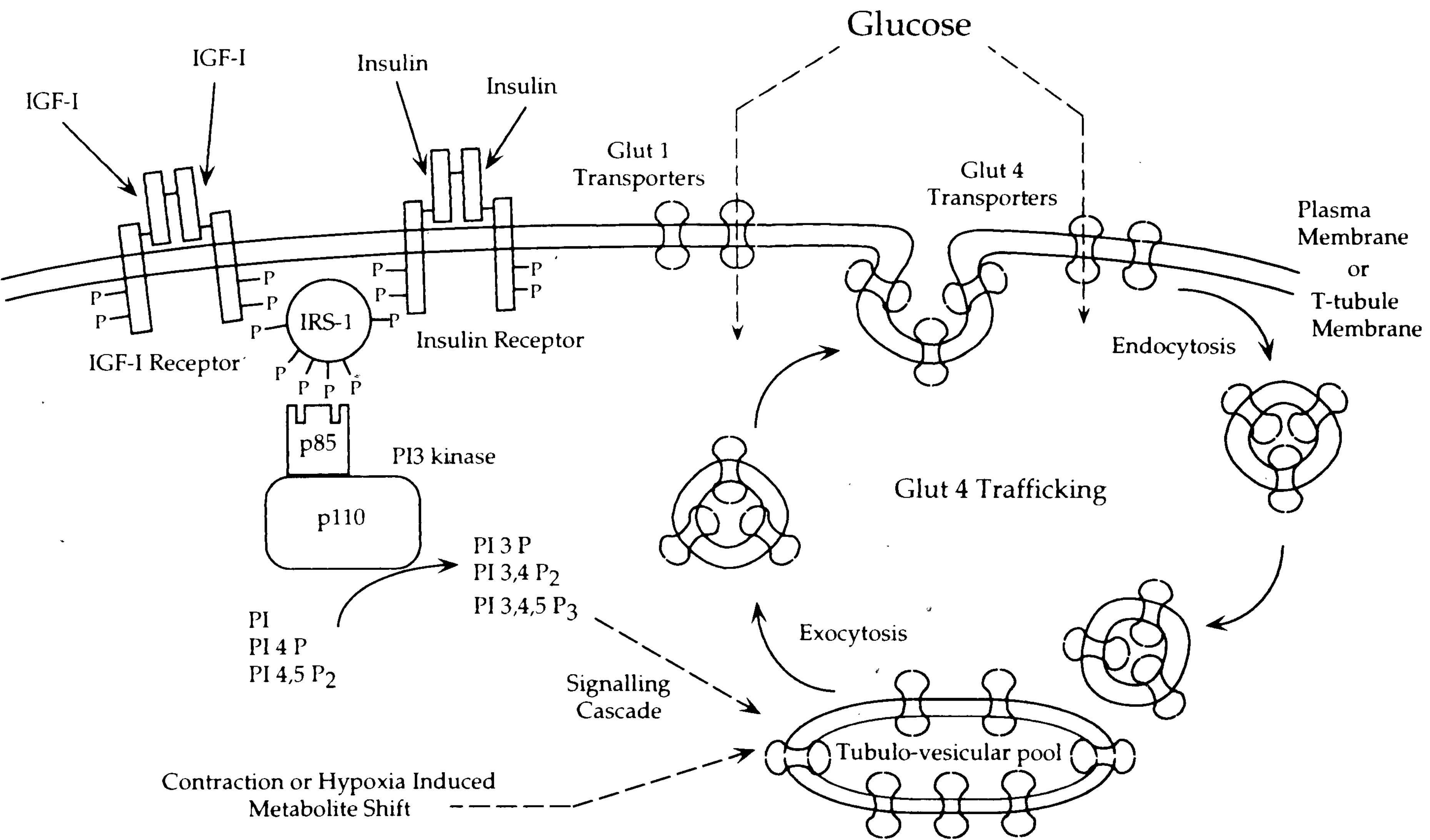

Figure 1.1: Schematic representation of the muscle glucose transport system

THE EFFECTS OF AGE ON MYOCARDIAL GLUCOSE TRANSPORT SYSTEM RELATED PROTEINS OF THE MOUSE

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Chapter 2

THE EFFECTS OF AGE ON MYOCARDIAL GLUCOSE TRANSPORT SYSTEM RELATED PROTEINS OF THE MOUSE

Abstract

This study examined the effects of aging on the myocardial protein content of Glut 4, Glut 1, insulin receptor, IGF-1 receptor, and PI3 kinase of the C57Bl/6 mouse. Insulin and IGF-1 receptor autophosphorylation activity was also assessed. Glut 4 content was increased fourfold between 22.5 and 29 mo. of age, as compared to 5 mo. animals. Glut 1 content was increased by 40% at 17 mo., returning to 5 mo. levels at 29 mo. Insulin and IGF-1 receptor content decreased early between 5 and 17 mo. by 20 to 35%, and remained constant until 29 mo. and 25 mo. respectively. Between 25 and 29 mo., IGF-1 protein levels decreased to 24% of 5 mo. control. Receptor autophosphorylation was increased at basal and maximally stimulating (10^{-6} M) concentrations of insulin or IGF-1 by two to fourfold. PI3 kinase content decreased 30% by 12 mo. and remained constant thereafter. We conclude that the components of the myocardial glucose transport system studied here are upregulated with advancing age in accordance to the increased importance of cardiac glucose metabolism with aging.

Keywords: heart, glucose transport, aging

2.1 Introduction

Aging is associated with the development of insulin resistance in peripheral tissues [10][5]. The major site for insulin mediated glucose disposal is skeletal muscle [10]. It is unknown whether the myocardium, which is also an insulin sensitive tissue, develops insulin resistance with advancing age, but there is evidence of cardiac insulin resistance in diabetic states [6].

Cardiac muscle is a highly oxidative tissue, and in the aerobically perfused heart, free fatty acid catabolism provides the major source of energy for ATP resynthesis, accounting for between 60 and 70% under basal conditions, with glucose and lactate metabolism providing the rest [21]. However, the contribution of free fatty acid oxidation to overall cardiac metabolism decreases with increasing age [1][9] and thus the importance of glucose oxidation and glycogenolysis increases in terms of energy provision [14].

While of quantitatively lesser importance than skeletal muscle for glucose disposal, age-related insulin resistance of the heart is nevertheless a serious concern in different pathological conditions during which glucose can become a more important metabolic fuel than fatty acids: 1) In responding to acute energy deficit, the working heart cannot rapidly increase uptake and metabolism of free fatty acids and relies on increased glycolysis and glucose oxidation brought about by accelerated glucose transport and glycogenolysis [6]; 2) Similarly, in conditions of chronic increase in workload, such as hypertension and the associated concentric hypertrophy, there is an increase in glucose uptake and a corresponding decrease in free fatty acid utilization [20][2]; 3) In the extreme case of myocardial ischemia, the heart must rely primarily on anaerobic glycolysis of exogenous glucose and endogenous

glycogen as oxygen deprivation renders the myocardium incapable of fatty acid β oxidation [17]. During severe ischemia, cardiac glycogen stores can be depleted within 1-2 hours [18] and thus any process that limits exogenous glucose availability, such as a compromised glucose transport system, can adversely affect myocardial function and survival.

Very little is known about whether the myocardial glucose transport system experiences age-related changes and develops insulin resistance similar to its skeletal muscle counterpart. Few studies have attempted to document changes in various components of the system during aging. Only Glut 4 content has been quantified, and was reported to undergo a small age-related decrease in rats[3][8]. If the myocardial glucose transport system does behave like the skeletal muscle system, then the heart may become compromised in the face of a metabolic challenge.

The purpose of the present study was to quantify age-related changes in several components of the myocardial glucose transport system in an attempt to document whether this system may be adversely affected by the normal aging process. Specifically, content of Glut 4 and Glut 1 transporters, insulin and IGF-1 receptors, and the signalling intermediate PI3 kinase were assessed by immunoblot. The function of the insulin and IGF-1 receptors was assessed by autophosphorylation assay.

2.2 Methods

Animals

Female C57Bl/6 mice aged 5, 12, 17, 22.5, 25, and 29 months (n=8 per group) were used for this study. All animals were obtained from the National Institute of Aging animal colony and group housed (n=3-8) in conventional cages under laminar air flow. Animals were maintained on a 12:12 hr light-dark cycle and provided with food (Purina Rodent Lab Diet 5001, Illinois, USA) and water ad libitum. All experiments were conducted at the same time of day following a 12 hour fast. Treatment of animals was certified by Simon Fraser University's animal care ethics committee, and all practices conformed to the regulations of the Canadian Council of Animal Care.

Tissue preparation

Hearts were removed under sodium pentobarbital anesthesia (50 mg/kg), blotted, immediately frozen in liquid nitrogen, and stored at -80°C until analysis. Hearts were weighed and homogenized in 15 volumes of ice-cold buffer containing 100 mM HEPES, pH 7.6; 150 mM NaCl; 5 mM EDTA; 5 mM $MgCl_2$; 2% Nonidet P-40; 1% Triton X-100; the following protease inhibitors: 2 mM phenylmethylsulfonyl fluoride (PMSF), 60 µM leupeptin, 60 µM pepstatin, 100 IU/ml bacitracin, 3 mg/ml aprotinin, 1 mg/ml benzamidine, 1 mg/ml N∝-p-tosyl-l-arginine methyl ester (TAME), 1 mg/ml N∝-benzoyl-l-arginine ethyl ester (BAEE); and the following phosphatase inhibitors: 100 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate. The homogenate was centrifuged at 10 000 g for 20 min at 4°C and the resulting supernatant was centrifuged at 150 000 g for 60 min at 4°C. The protein content of the final supernatant was determined by

Bradford protein assay (Bio-Rad) using BSA standards. All samples were within the linear range of 2-12 μg. Crude solubilized membrane extracts were kept frozen at -80°C until further analysis.

Western blots

A crude membrane preparation containing 200 μg of protein was mixed with sample buffer containing 60 mM Tris, pH 6.8; 2% wt/vol SDS; 10% vol/vol glycerol, 5% vol/vol β-mercaptoethanol to a final volume of 125μl, boiled for 5 min and separated by SDS-PAGE on a 10% acrylamide 0.1% bis-acrylamide gel. Proteins were electrotransferred 3 hrs at 60V to 0.45 μm PVDF membrane (Millipore), visualized by Ponceau Red (0.2% in 3% TCA, Sigma) and washed in TBST (50 mM Tris, pH 7.4; 150 mM NaCl; 0.5% Triton X-100). Membranes were blocked in TBST containing 3% BSA, washed in TBST and incubated overnight at 4°C in primary antibody. Membranes were washed in TBST, incubated in HRP-conjugated secondary antibody 90 min at 25°C and washed in TBST followed by TBS (50 mM Tris, pH 7.4; 150 mM NaCl). Membranes were immersed in Enhanced Chemi-Luminescence reagent (Amersham) and exposed to Hyperfilm (Amersham). Developed films were quantified by laser densitometry using an LKB UltroScan XL densitometer and GelScan XL software. In order to allow comparisons across age groups, all samples were subjected to electrophoresis simultaneously using 6 gels. The gels were cut above and below the molecular weight of interest and transferred simultaneously onto a single membrane.

Except where indicated, all antibodies were obtained from Santa Cruz Biotechnology. The following primary antibodies and concentrations were used for immunoblotting: goat-polyclonal anti-GLUT-4, 1:5000; rabbit polyclonal anti-GLUT-1, 1:3333 (East-Acres Biologicals); mouse monoclonal

anti-insulin receptor β-subunit, 1:10 000; rabbit polyclonal anti-IGF-1 β-subunit, 1:5000; rabbit polyclonal anti-p85 α-subunit, 1:5000. All primary antibodies were prepared in TBST, 1% BSA, and 0.5% sodium azide.

The following HRP-conjugated secondary antibodies and concentrations were used for immunoblotting: donkey anti-goat, 1:30 000; goat anti-rabbit, 1:30 000; goat anti-mouse, 1:30 000. All secondary antibodies were prepared freshly in TBST and 1% BSA.

Receptor autophosphorylation assay

For six samples from both the 12 and the 29 month age groups, 60 µl of crude membrane preparation containing 200 µg of protein was incubated 18 hrs at 4°C with 20 µl of either insulin or IGF-1 diluted to a final concentration ranging from 1×10^{-11} M to 1×10^{-6} M and with 20 µl of 5x concentrate assay buffer (50 mM HEPES, pH 7.4; 5 mM $MnCl_2$; 0.1% BSA; 0.1% Triton X-100; 100 µM sodium orthovanadate) in a final volume of 100µl. The phosphorylation reaction was activated by adding 10 µl of 10x concentrate 1 mM ATP/ 10 mM $MgCl_2$ solution and allowed to proceed 60 minutes at 4°C before being terminated by denaturation of the proteins following the addition of 50 µl of 3x concentrate sample buffer and boiling for 5 min. Boiled samples were subjected to SDS-PAGE and western blot analysis, as described above. Tyrosine phosphorylation of the β subunits of both insulin and IGF-1 receptors co-migrating at 97 kDa, was measured by probing blots with mouse monoclonal anti-phosphotyrosine antibody (Santa Cruz) at a concentration of 1: 1000. Phosphorylation at 1×10^{-11} M ligand was considered basal and phosphorylation at 1×10^{-6} M ligand was considered maximal, based on dose-response experiments. Insulin-stimulated phosphorylation results were

normalized by insulin receptor content, while IGF-1 stimulated phosphorylation results were normalized by IGF-1 receptor content.

Statistical Analysis

Statistical comparisons of western blot analyses between 6 age groups were performed by one-way ANOVA for each dependent variable. Significance was set at the 0.05 level. Significant differences were analyzed post-hoc using a Duncan test. Comparisons of autophosphorylation assay results between 2 groups were performed by paired T-test, with significance set at the 0.05 level. Pairwise correlations between all variables were calculated with significance set at the 0.05 level. All statistical analysis was performed using SPSS 6.1 software for Macintosh.

2.3 Results

Descriptive data for the various age groups is contained in Table 2.1. Heart mass demonstrated an increasing trend across the lifespan of the animal while body weight remained constant after 17 months of age. Heart mass and body weight were significantly different from 5 month control animals in 22.5, 25 and 29 month animals. Heart mass to body weight ratio was significantly increased at 25 and 29 month, as compared to 5 month animals. Soleus muscle mass was significantly greater at 12 months than at all other age points recorded. Specific protein levels are expressed relative to 5 month old animal values and are outlined in Table 2.2 and illustrated in Figure 2.1 expressed per μg protein and in Figure 2.2 expressed per mg wet tissue. No differences in protein yields were observed as a function of age (Table 2.2).

Significant age-related changes were observed for all five glucose transport system related proteins studied. Myocardial Glut 4 protein content exhibited an increasing trend between 5 and 25 months of age (Figure 2.1A). This trend was observed despite the exclusion of some samples which clearly over-expressed Glut 4 (Figure 2.3A). About 10 percent of the samples from 17 to 29 month old animals over-expressed Glut 4. By 22.5 months of age, Glut 4 protein levels were significantly increased to over four times the amount of the 5 month control group, peaking at 25 months (5.3 fold greater than 5 month control) and decreasing at 29 months to levels similar to 22.5 month old animals. This pattern of increase was not parallelled by the other proteins of interest (Table 2.2).

Myocardial Glut 1 transporter protein content was found to be elevated 25 to 40% in the 17 to 25 month old animals (Figure 2.1B). However, by 29 months of age, Glut 1 levels had returned to basal 5 month old levels. Insulin receptor protein levels showed an early decreasing trend between 5 and 17 months becoming significant at 17 months, with no further change thereafter to 29 months (Figure 2.1C). From 17 months on, levels ranged from 69% to 58% of 5 month animals. Similarly, IGF-1 receptor protein levels were observed to fall to 77% of 5 month controls at 12 months and to remain constant up to 25 months of age (Figure 2.1D). However, between 25 and 29 months of age, the IGF-1 receptor levels fell precipitously to 24% of control values. PI3 kinase protein levels were also shown to drop significantly to 70 percent at 12 months of age and to continue to hover between 70 and 60% of control for the remainder of the mouse lifespan (Figure 2.2E). None of the other proteins measured in the five samples over-expressing Glut 4 demonstrated significant deviations from those values observed for the non-over expressing samples (Figure 2.3B).

Significant age-related changes in insulin and IGF-1 receptor β-subunit autophosphorylation were also observed between the 12 and 29 month old animals (Table 2.3). Phosphorylation of the 97 kDa band on immunoblots of crude membrane extracts, corresponding to the β-subunit of both receptors, was measured after stimulation by either insulin or IGF-1. When results were normalized for insulin receptor content, β-subunit phosphorylation in the 29 month group was shown to be slightly less than double that of the 12 month group at a basal insulin concentration of 1×10^{-11} M. At a maximal insulin concentration of 1×10^{-6} M, this significant age related difference became slightly more than double. Similar results were observed during IGF-1 stimulation after normalization for IGF-1 receptor number. At 1×10^{-11} M IGF-1, there was a significant fourfold increase in β-subunit phosphorylation in the 29 month group as compared to the 12 month group. At a maximal 1×10^{-6} M dose of IGF-1, this difference dropped to slightly greater than threefold. However, despite this 3 fold greater maximal autophosphorylation of the IGF-1 receptor in the aged animals, the percentage increase from basal to maximal phosphorylation was about half that of the young mature animals, although this difference did not reach significance.

Significant positive correlations were observed between IGF-1 receptor and insulin receptor protein content (Figure 2.4A) and between PI3 kinase protein levels and insulin receptor content (Figure 2.4B). Significant negative correlations were observed between PI3 kinase and Glut 4 protein content (Figure 2.4C) and between Glut 4 and insulin receptor levels (Figure 2.4D). A significant relationship was observed between insulin receptor, Glut 4 and PI3 kinase levels (Figure 2.4E). Results are summarized in Table 2.4.

2.4 Discussion

The results of the present study have yielded a number of new observations. It appears that the C57Bl/6 mouse myocardium undergoes many age-related changes to its glucose transport system related proteins between the ages of 5 months, considered young mature, and 29 months, considered senescent. Of the five proteins studied here, only the Glut 4 transporter had previously been examined as part of an aging study of cardiac tissue. Cartee [3] reported small Glut 4 protein content decreases in Fisher 344 rat cardiac tissue of 15% between the ages of 3.5 and 13 and of an additional 12% between 13 and 25 months of age. Similar numbers were reported by Hall [8], who also showed no attenuation of these age-related changes with 10 weeks of low intensity exercise training in Sprague-Dawley rats. These findings are in contrast to the skeletal muscle literature which reports all age-related changes in muscle Glut 4 content before the age of 12 months, with no further drop thereafter [7]. Contrary to the above findings, our results do not indicate an age-related decrease in myocardial Glut 4, but rather a very large increase becoming significant at 22 months of age and peaking at 25 months. Ages can be compared directly between the rat model and the mouse model as the lifespan of both species is slightly more than 30 months. Our results were confirmed by reprobing our immunoblots with other Glut 4 antibodies (data not reported). A small number of samples found in the older age groups greatly over-expressed Glut 4 protein and were excluded from the analysis. However, these samples did not differ from other samples of the same age group for any other protein measurement.

An age-related increase in myocardial Glut 4 protein content can be the result of increased translational rate, increased stability, or decreased protein turnover. Such an increase in protein content may be explained by the well

documented decrease in cardiac free fatty acid metabolism observed with advancing age [1][9]. A corresponding increase in glucose metabolism [14] could be expected to be paralleled by an increase in Glut 4 transporter content, as facilitated diffusion of glucose into the cell is thought to be the rate limiting step to glycolysis of exogenous glucose [11]. However, even a doubling of cardiac glucose metabolism would not account for the shear magnitude of increase in Glut 4 content.

The discrepancies between our findings and those of others may be due to inter-species differences, as most studies of the glucose transport system have used a rat model. The C57 mouse model chosen for this study is extensively employed for aging studies, however, important differences do exist between this animal model and rat models more commonly employed. Most importantly, and in direct contrast to the Fisher 344 and Sprague-Dawley models, the C57 maintains a constant body weight after 12 months of age and exhibits a relatively high level of intrinsic activity throughout its lifespan.

Discrepancies could also be due to methodology. In order to isolate membrane receptors as well as transporters from less than 100 mg of cardiac tissue, we prepared crude solubilized membrane extracts. Glut 4 has traditionally been studied in whole cell homogenates using a sucrose buffer. However, it is unlikely that we would lose a significant amount of transporters due to our fractionation, as intracellular Glut 4 is thought to be associated with vesicular structures. Furthermore, analysis of discarded tissue revealed negligible Glut 4 loss.

An age-related increase in cardiac glucose metabolism would also explain our findings of increased myocardial Glut 1 transporter content of ~30-40% between 17 and 25 months of age as compared to 5 month levels.

Glut 1 is believed to be involved in basal glucose transport [15]. Skeletal muscle expresses Glut 1 in very low levels as compared to cardiac muscle [12] where basal transport is quantitatively much more important. The trend of Glut 1 increase, while not of the same magnitude, roughly parallels the trend of Glut 4 increase. Glut 1 content returns to 5 month levels at 29 months, at which age a decrease in Glut 4 to 22.5 month levels was also observed. This is suggestive of co-regulation of these two transporter isoforms.

Like the transporters, the insulin and the IGF-1 receptor protein contents follow relatively similar trends over the lifespan of the mouse. Both receptor contents decreased early by 25 to 35% between 5 and 17 months, perhaps reflecting an overall slowing of the animal's metabolism. Thereafter, levels stayed relatively constant up to 29 months for the insulin receptor and up to 25 months for the IGF-1 receptor, after which point levels fell dramatically to ~25% of 5 month levels. The IGF-1 receptor is thought to be more important for mitogenic signalling than the insulin receptor [4], hence this precipitous drop may be a reflection of a downregulation of mitogenic activity in the senescent animal. Furthermore, the increased importance of cardiac glucose metabolism with advancing age would require that insulin receptor content be maintained. However, a postulate of decreased mitogenic signalling is not supported by the observation of cardiac hypertrophy (discussed below) between 25 and 29 months of age. Another explanation for the sharp decrease between 25 and 29 months observed only in IGF-1 receptors would be an increase in hybrid receptor content. Hybrid receptors contain an αβ-heterodimer from an insulin receptor and one from an IGF-1 receptor [19] and thus, such an increase would mask a parallel decrease in both insulin and IGF-1 receptor contents. Hybrids have been shown to function as IGF-1 receptors, however their physiological relevance is still unclear [19].

While protein content of the receptors was observed to be decreased after 17 months, receptor function as assessed by autophosphorylation assay, appeared to be enhanced in old animals. Basal and maximally insulin-stimulated or maximally IGF-1-stimulated receptor β-subunit phosphorylation were markedly increased in 29 month old animals as compared to 12 month old when values were normalized for receptor content as determined by immunoblot. However, under IGF-1 stimulation, the response range underwent a decreasing trend suggesting the presence of constituvely active IGF-1 receptors in the older animals. No attempt was made to isolate the two receptors since it was assumed that cross-reactivity is minimal [13] and since the activation of both receptors initiates the same metabolic signalling cascade. Receptor autophosphorylation is thought to reflect tyrosine kinase activity and thus signalling potential of the receptor [23], and measurement does not require the use of exogenous substrate. Since receptor content is not increased in the face of the increased importance of cardiac glucose metabolism in old animals, an increase in receptor activity is an alternate form of upregulation of signal transduction potential.

The signalling intermediate PI3 kinase was examined as a possible site of regulation within the metabolic signalling cascade initiated by the receptors. Not surprisingly, its initial decrease in content between 5 and 12 months followed by stable levels thereafter, mirrors receptor content very closely. PI3 kinase's activity is dependent on tyrosine phosphorylation of IRS-1 [16], which in turn is controlled by receptor activity [22]. Therefore the activity of PI3 kinase, although not measured, should not be expected to decrease while its protein content is stable and receptor function is upregulated.

The behaviour of all five myocardial glucose transport system related proteins suggests an upregulation of the glucose transport system which would concord with an age-related increase in cardiac glucose metabolism. It has also been reported that a shift in cardiac fuel preference away from FFA oxidation occurs with pressure-overload cardiac hypertrophy [2]. We observed an important increasing trend in heart mass and heart to body weight ratio with advancing age in our animals. No attempt was made to characterize this hypertrophy histologically, however no change in protein yield was observed. The C57Bl/6 mouse is not known to be a spontaneously hypertensive animal model, and thus the observed hypertrophy is difficult to explain. Furthermore, soleus muscle atrophy in older animals would suggest that the observed cardiac hypertrophy is not due to increased levels of circulating anabolic hormones and that the C57 is a normal aging model. However, an increased importance of glucose metabolism in the hypertrophied heart could also be expected to cause an upregulation of the glucose transport system.

We conclude that despite early decreases in insulin and IGF-1 receptor contents and PI3 kinase content, the components of the myocardial glucose transport system studied here are upregulated with aging, in accordance with the documented increase in myocardial reliance on exogenous glucose in older animals. This is supported by increases in transporter content and receptor function accompanied by relatively stable receptor and signalling intermediate content with advancing age.

2.5 Acknowledgements

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2.6 References

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2.7 Tables and figures

Table 2.1: Descriptive data for age groups

Age (mo)	Sample Size (n=)	Heart Mass (mg)	Body Weight (g)	Heart / Body Weight Ratio	Soleus Mass (mg)
5	8	100.9 ±12.4	22.7 ±1.1	0.45%	7.2 ±0.8
12	8	108.7 ±9.1	30.9 ±3.5[a]	0.35%	8.8 ±0.9[b]
17	8	117.2 ±7.9	24.9 ±2.2	0.47%	na
22.5	8	142.0 ±12.0[a]	28.6 ±1.9[a]	0.50%	7.5 ±0.4
25	8	153.0 ±26.5[a]	27.6 ±5.9[a]	0.56%[a]	7.2 ±1.3
29	8	165.6 ±32.0[a]	28.2 ±1.5[a]	0.59%[a]	na

data expressed as mean ± SD

a = different from 5 mo. control ($p<0.05$)
b = different from all groups ($p<0.05$)

Table 2.2: Summary of western blot results

	Age (mo) 5	12	17	22.5	25	29
Crude protein yield	12.0% ±1.2	12.5% ±1.3	12.1% ±1.0	13.1% ±1.0	12.2% ±0.7	11.9% ±0.9
Glut-4						
% control/200μg protein	100 ±41.3	124.5 ±80.6	187.3 ±88.2	422.2 ±240.8[a]	530.5 ±268.6[a]	415.0 ±281.1[a]
% control/mg wet tissue	100 ±49.2	124.9 ±81.3	186.0 ±83.9	442.5 ±239.0[a]	527.6 ±253.7[a]	410.6 ±300.8[a]
	*		*	*	*	*
Glut-1						
% control/200μg protein	100 ±21.6	102.8 ±28.8	140.0 ±24.1[ab]	126.8 ±21.3	136.4 ±23.8[b]	95.1 ±53.8
% control/mg wet tissue	100 ±23.6	103.0 ±34.3	142.2 ±30.8[ab]	138.1 ±19.7[b]	139.5 ±24.3[ab]	93.9 ±50.1
	*	*				
Insulin Receptor						
% control/200μg protein	100 ±14.2	93.8 ±12.5	67.1 ±14.3[a]	68.7 ±9.5[a]	58.5 ±6.8[a]	57.6 ±5.3[a]
% control/mg wet tissue	100 ± 6.9	98.1 ±5.0	67.3 ±9.8[ab]	75.4 ±8.5[ab]	60.1 ±5.3[a]	58.0 ±7.3[a]
IGF-1 Receptor						
% control/200μg protein	100 ±16.0	76.9 ±11.2[ab]	79.1 ±14.7[ab]	76.2 ±12.2[ab]	75.5 ±12.2[ab]	24.0 ±29.9[a]
% control/mg wet tissue	100 ±11.7	81.8 ±9.0[b]	80.7 ±9.8[b]	85.0 ±12.1[b]	79.0 ±12.9[b]	26.1 ±33.1[a]
	*					
PI3 Kinase						
% control/200μg protein	100 ±17.9	70.7 ±20.2[a]	68.4 ±18.7[a]	62.1 ±9.3[a]	60.0 ±8.2[a]	65.8 ±18.6[a]
% control/mg wet tissue	100 ±18.4	74.5 ±24.6[a]	68.0 ±15.3[a]	67.7 ±8.7[a]	61.4 ±9.2[a]	65.2 ±16.2[a]

protein data expressed as mean ± SD normalized as % of 5 mo group
n=8 except where otherwise indicated by * (n=5-7)

a = different from 5 mo. control ($p<0.05$)
b = different from 29 mo. ($p<0.05$)

Table 2.2 Summary of western blot results for 5 proteins, expressed per 200 μg protein and per mg wet tissue

Table 2.3: Summary of receptor autophosphorylation assay results

		Age (mo)	
		12	**29**
Insulin Stimulation			
AU per 200 μg protein	Basal phosphorylation (10^{-11} M insulin)	0.193 ±0.081	0.227 ±0.109
	Maximal phosphorylation (10^{-6} M insulin)	0.519 ±0.287	0.711 ±0.417
AU per AU insulin receptor	Basal phosphorylation (10^{-11} M insulin)	0.173 ±0.073	0.331 ±0.159 *
	Maximal phosphorylation (10^{-6} M insulin)	0.463 ±0.257	1.035 ±0.606 *
	% Incremental increase	166.7% ±61.0	220.2% ±93.2
IGF-1 Stimulation			
AU per 200 μg protein	Basal phosphorylation (10^{-11} M IGF-1)	0.228 ±0.097	0.309 ±0.199
	Maximal phosphorylation (10^{-6} M IGF-1)	0.384 ±0.189	0.394 ±0.225
AU per AU IGF-1 receptor	Basal phosphorylation (10^{-11} M IGF-1)	0.223 ±0.095	0.968 ±0.624 *
	Maximal phosphorylation (10^{-6} M IGF-1)	0.375 ±0.185	1.234 ±0.706 *
	% Incremental increase	82.0% ±57.7	35.3% ±35.9

Data expressed as mean AU / 200 μg protein ± SD
Normalized data expressed as mean AU phosphorylation / AU receptor protein ± SD
n=6 for all measurements
* denotes significant difference from 12 mo. group ($p<0.05$)

Table 2.3 Insulin and IGF-1 stimulated receptor autophosphorylation as detected by western blot using PY69 anti-phosphotyrosine antibody

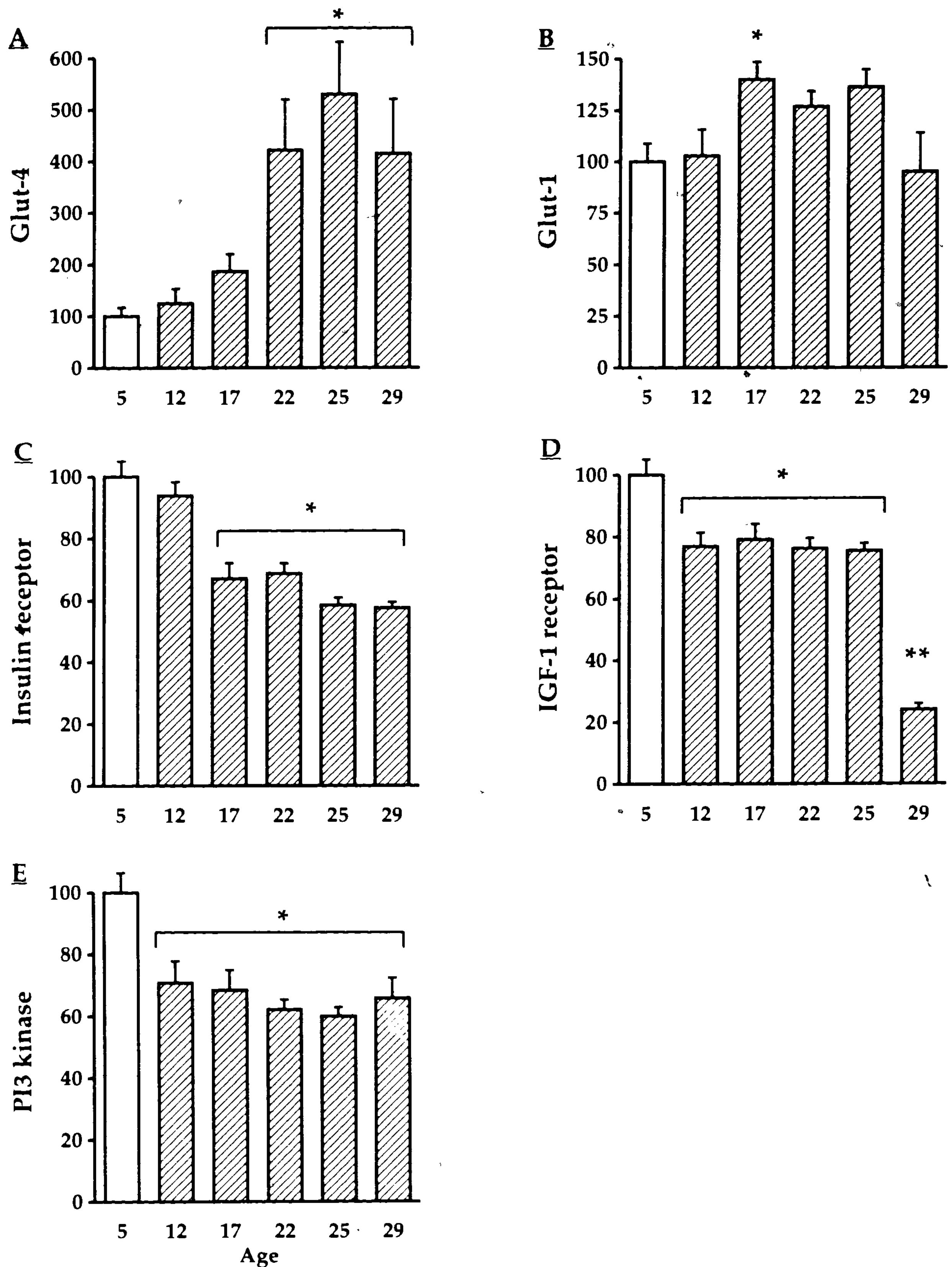
Table 2.4: Correlation table

	Glut-4	Glut-1	Insulin receptor	IGF-1 receptor	PI3 kinase
Glut-4	**1.000**				
Glut-1	**0.295** (38) p=0.072	**1.000**			
Insulin receptor	**-0.444 *** (41) p=0.004	**-0.238** (43) p=0.125	**1.000**		
IGF-1 receptor	**-0.210** (40) p=0.194	**-0.017** (42) p=0.913	**0.585 *** (47) p=0.000	**1.000**	
PI3 kinase	**-0.475 *** (41) p=0.002	**-0.239** (43) p=0.123	**0.529 *** (48) p=0.000	**0.232** (47) p=0.117	**1.000**

correlation coefficient / cases / 2 tailed significance
* denotes significance ($p<0.05$)

Table 2.4 Correlation table of western blot data for 5 proteins. Data points for all age groups correlated.

Figure 2.1: Graphical summary of western blot results expressed per 200 μg protein

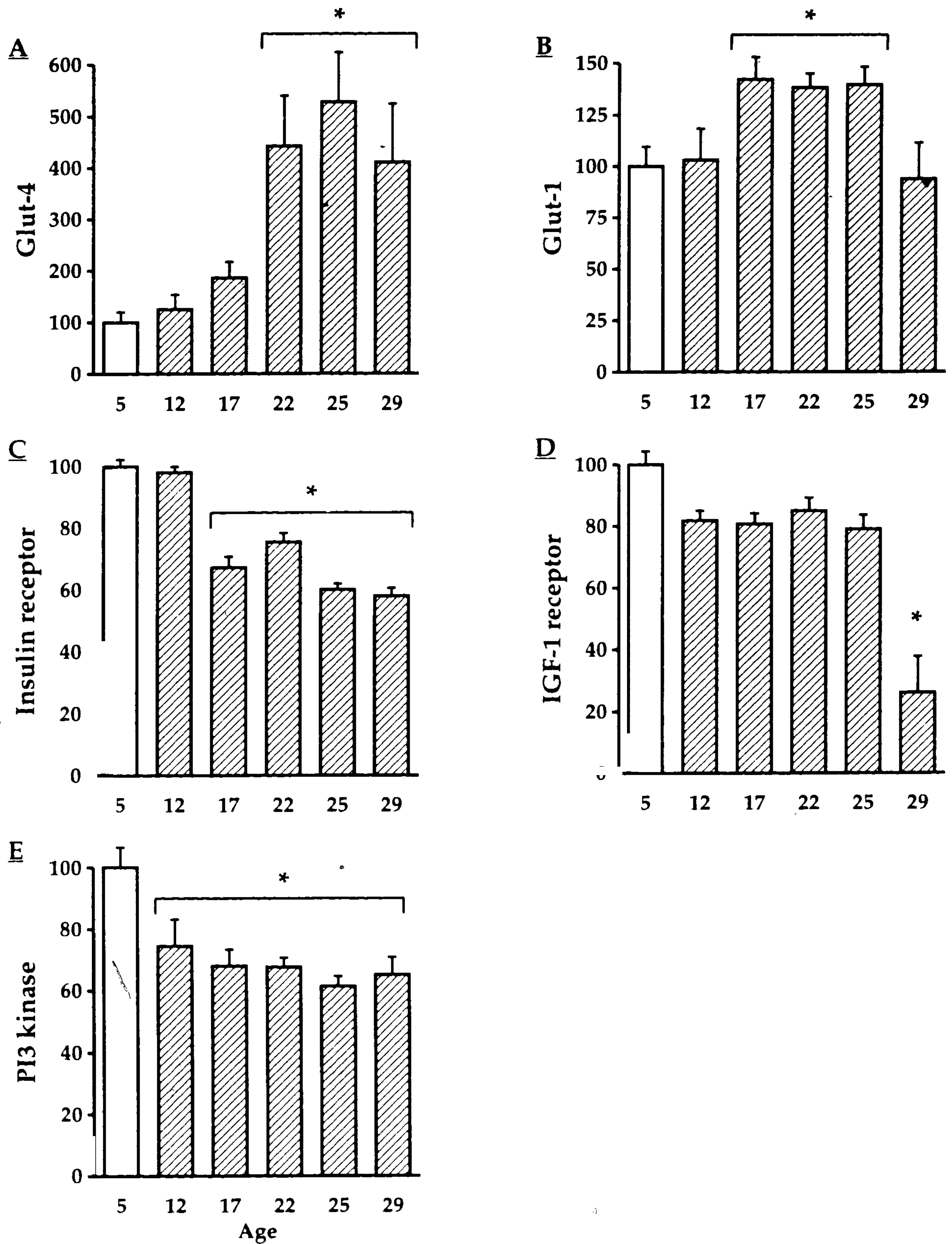


Data expressed as % of 5 month control group / 200 μg protein

* significantly different from 5 mo group ($p<0.05$)

** significantly different from all other groups ($p<0.05$)

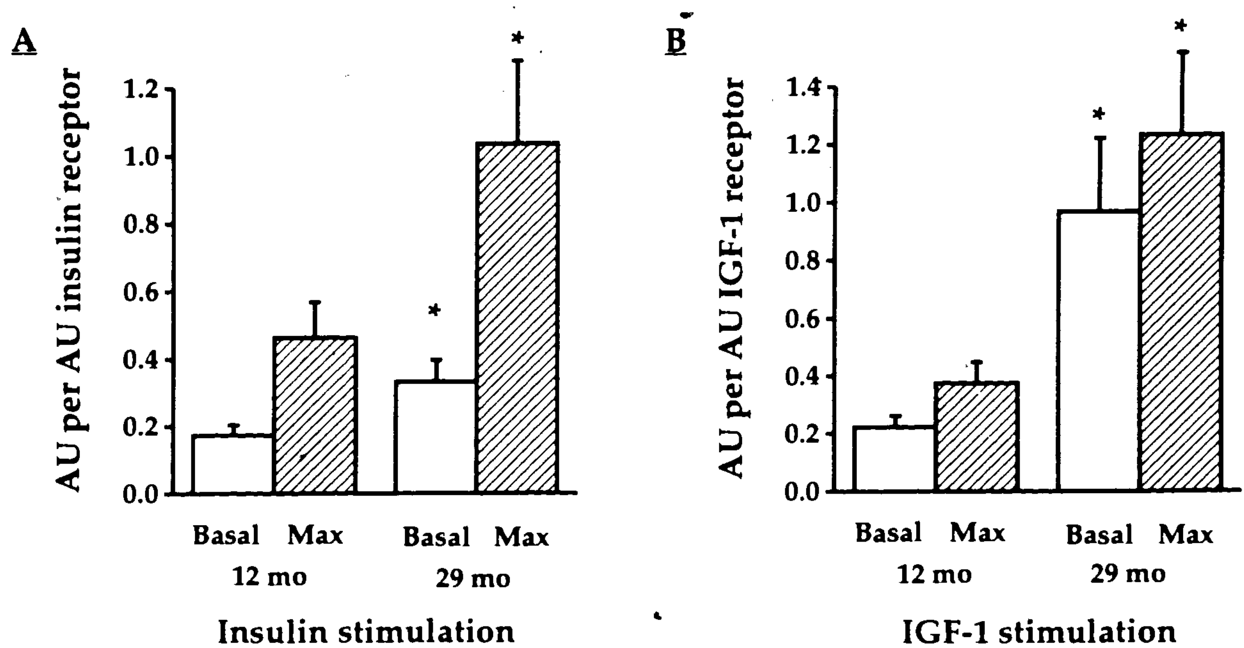
Figure 2.2: Graphical summary of western blot results expressed per mg wet tissue



Data expressed as % of 5 month control group / mg wet tissue

* significantly different from 5 mo group (p<0.05)

Figure 2.3: Graphical summary of autophosphorylation assay results



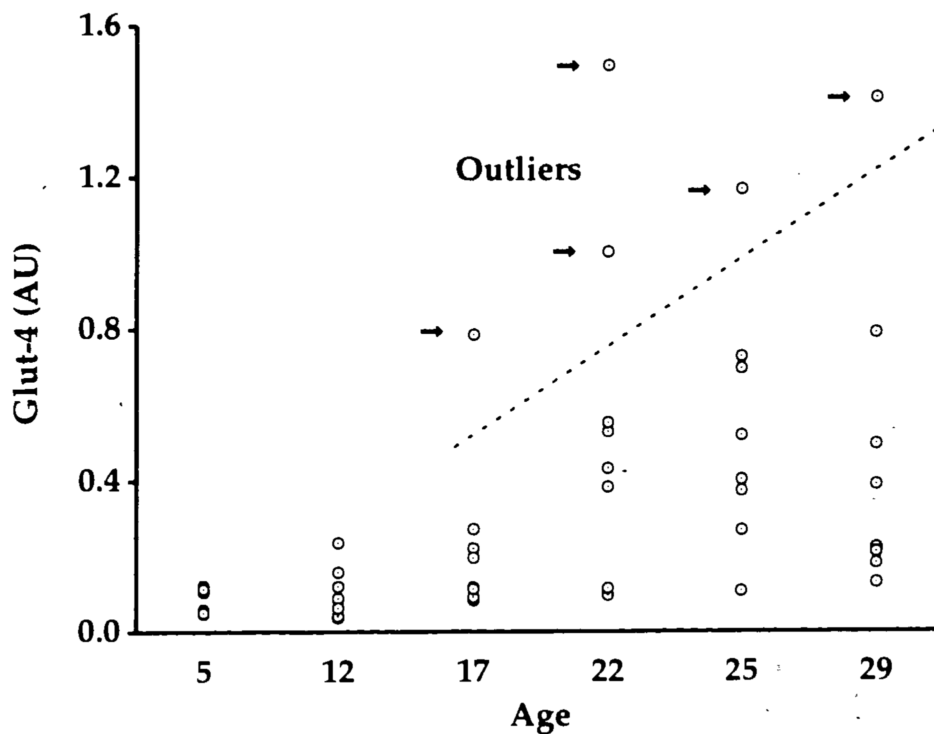
Data expressed as: A) mean AU phosphorylation per AU insulin receptor
B) mean AU phosphorylation per AU IGF-1 receptor

* denotes sinificant difference from 12 mo. group ($p<0.05$)

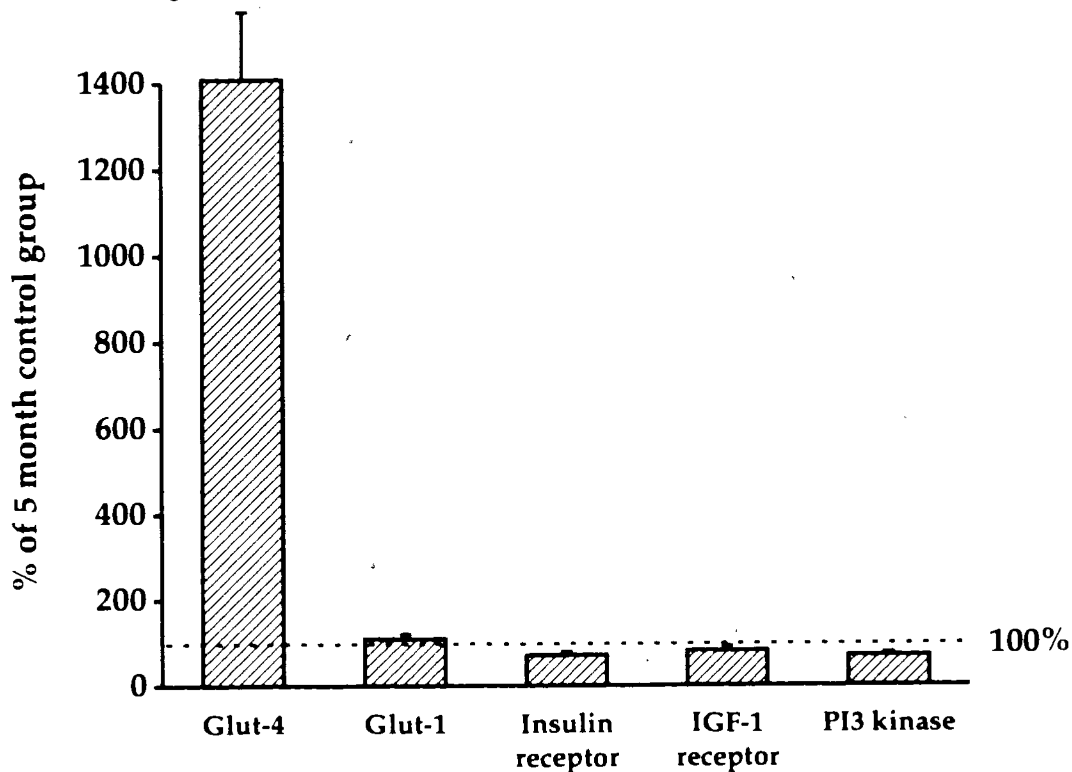
n=6 for all measurements

Figure 2.4: Graphical representation of samples overexpressing Glut-4

A Scatter of Glut-4 data points across age groups



B Corresponding change in other measurements for 5 samples overexpressing Glut-4



data expressed as (A) arbitrary units / 200 μg protein

(B) % of 5 month control group / 200 μg protein

Figure 2.5: Scatter diagrams for significant correlations

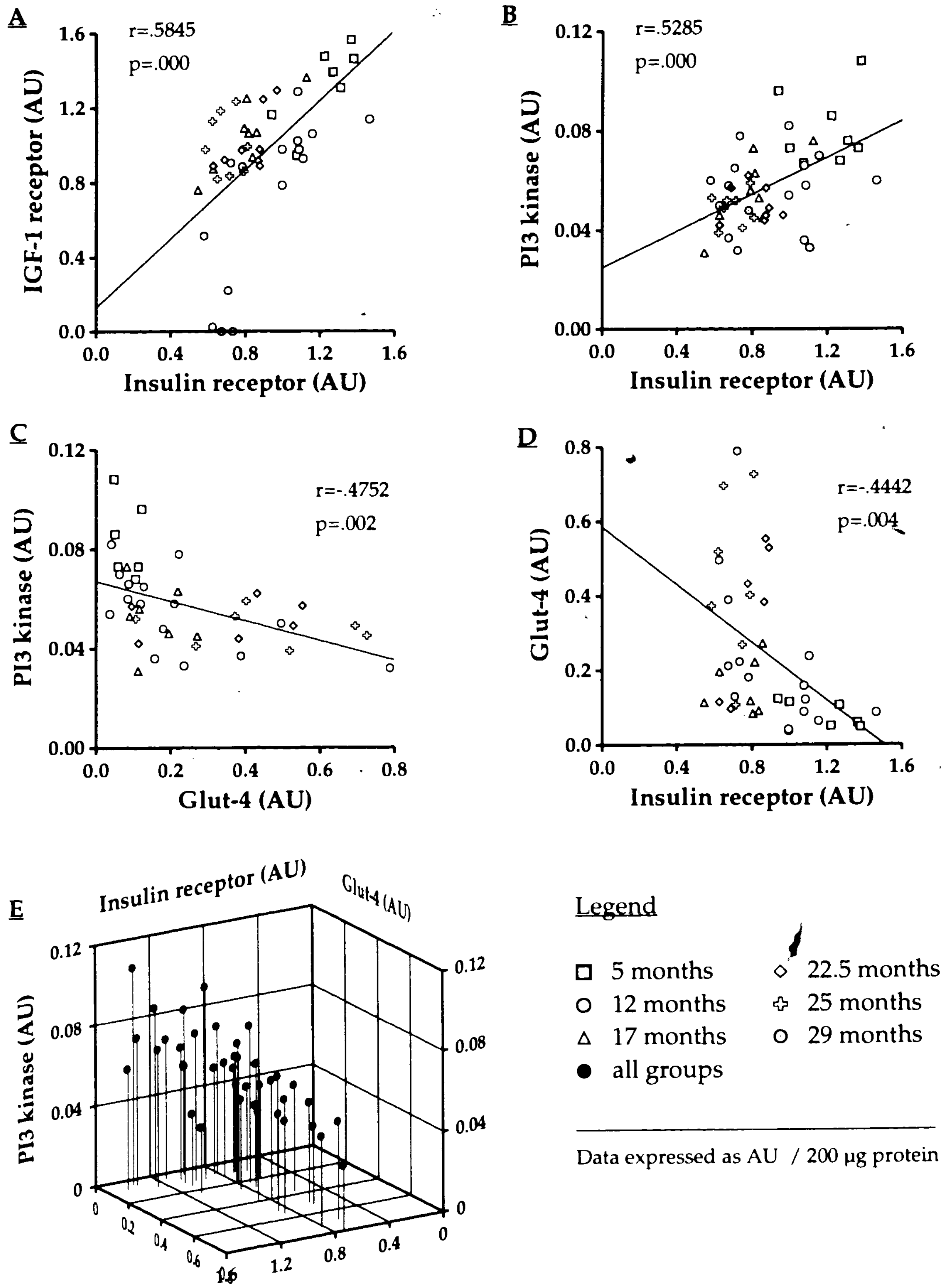
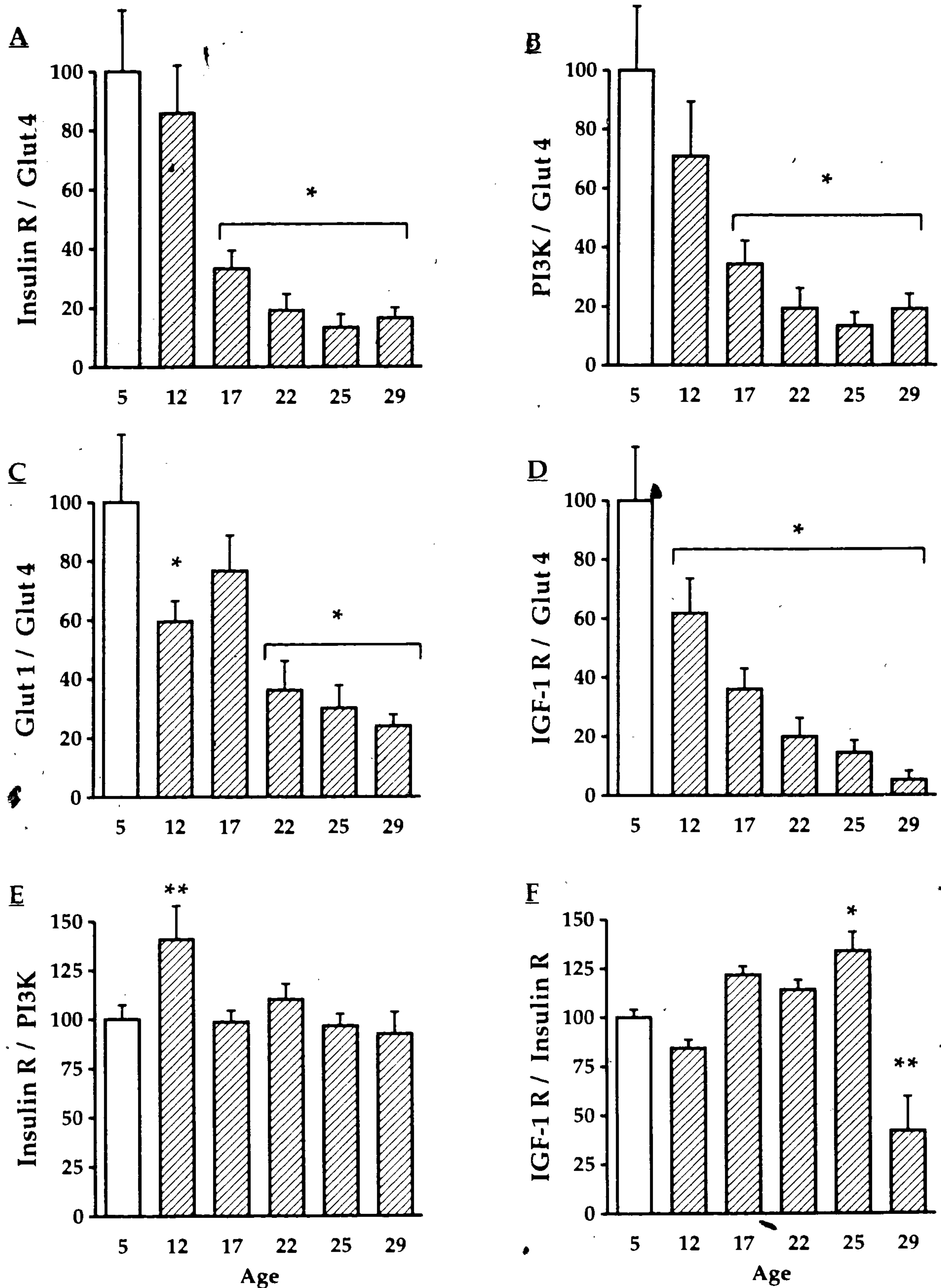


Figure 2.6: Graphical comparison of variable ratios across age groups

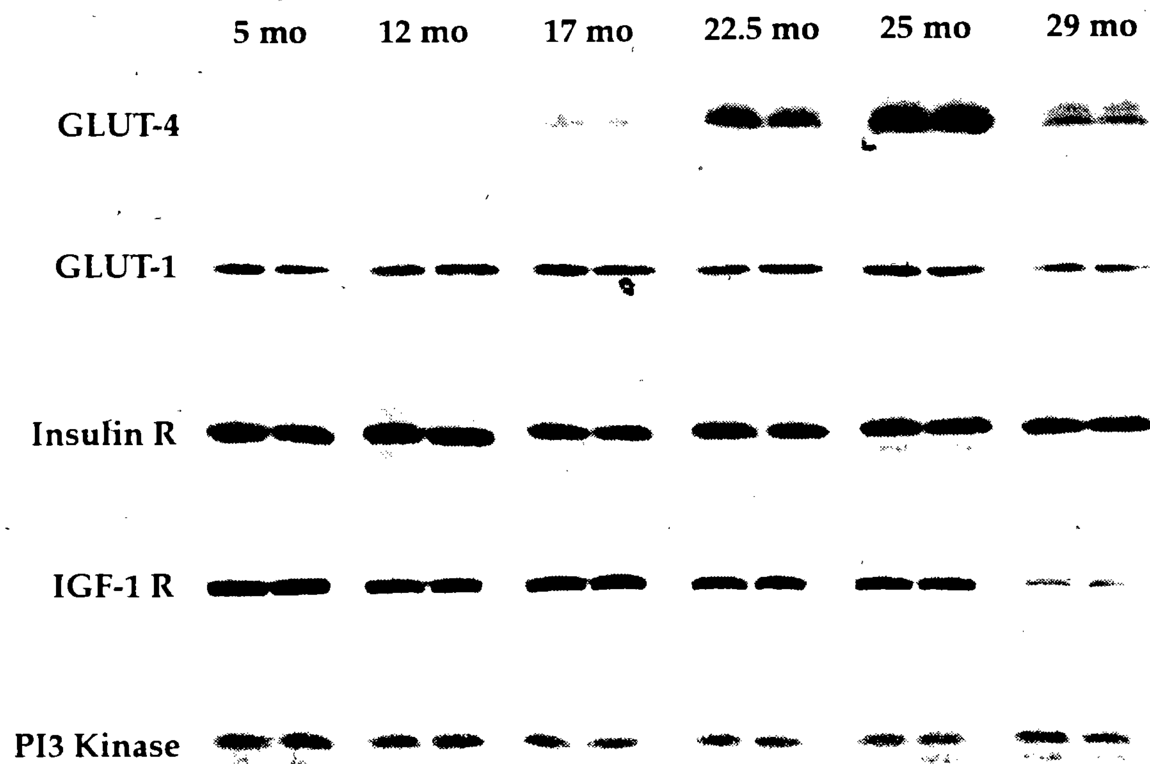


Data expressed as % of 5 month control group / 200 μg protein

* significantly different from 5 mo group ($p<0.05$)

** significantly different from all other groups ($p<0.05$)

Figure 2.7: Representative immunoblots



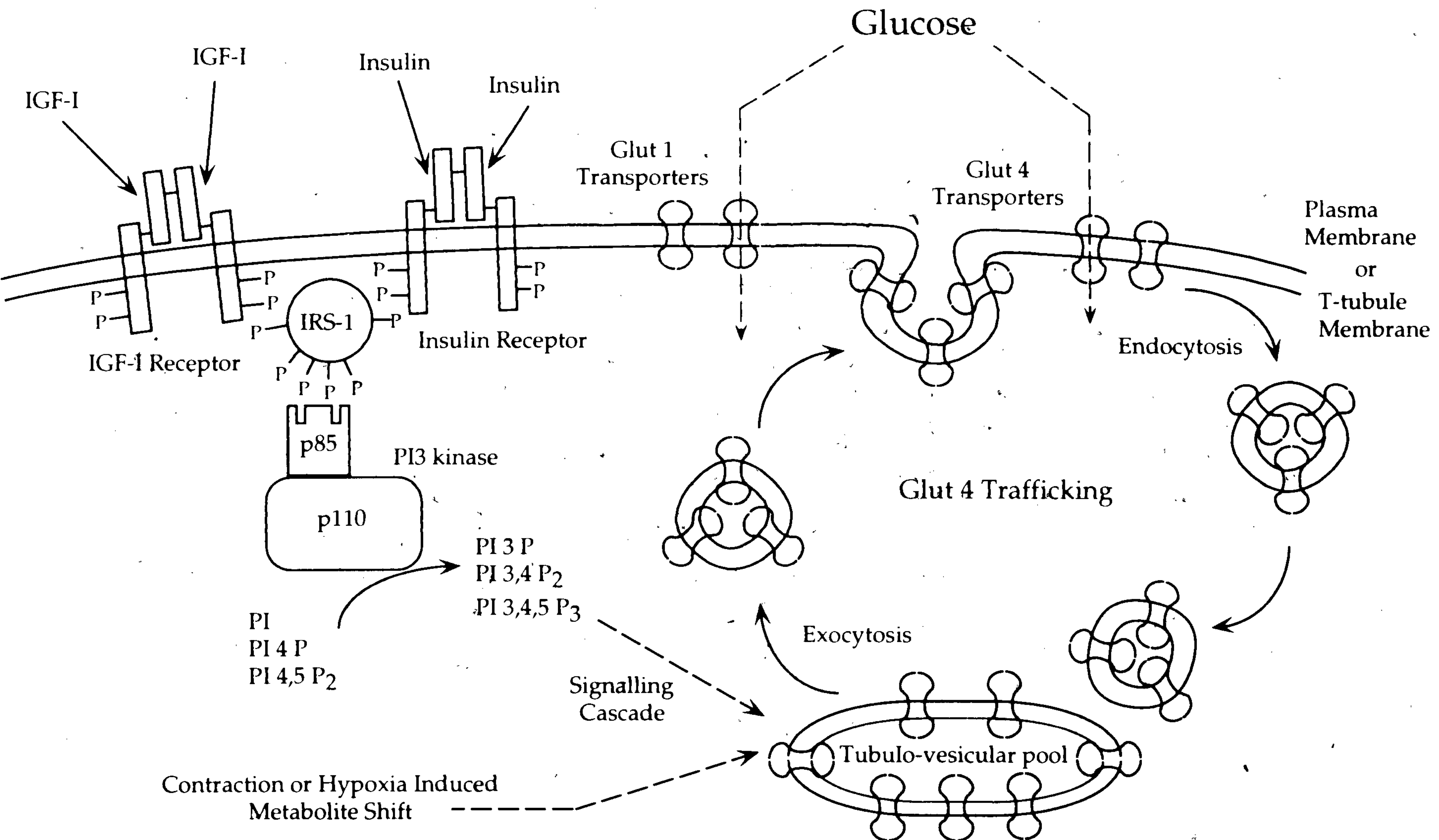


Figure 2.8: Schematic representation of the muscle glucose transport system

THE EFFECTS OF EXERCISE ON GLUCOSE TRANSPORT SYSTEM RELATED PROTEINS OF THE AGED MOUSE MYOCARDIUM

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Chapter 3

THE EFFECTS OF EXERCISE ON GLUCOSE TRANSPORT SYSTEM RELATED PROTEINS OF THE AGED MOUSE MYOCARDIUM

Abstract

The effects of 9 months of chronic voluntary exercise, 2.5 hours of forced acute exercise, and a combination of both treatments, on content of myocardial glucose transport system related proteins were assessed in 24 month old C57Bl/6 mice. Specifically, ventricular content of Glut 4 and Glut 1 transporters, insulin and IGF-1 receptors, and PI3 kinase was quantified by immunoblot. Chronic exercise was ineffective at altering the contents of any proteins, but potentiated the effects of acute exercise. Acute exercise in combination with chronic exercise caused increases of 114% in Glut 1 content and 33% in PI3 kinase content, expressed per mg wet tissue. Acute exercise alone or in combination with chronic exercise decreased insulin receptor content by 34% and 44% respectively. We conclude that the aged mouse myocardial glucose transport system retains the capacity to respond to exercise.

Keywords: heart, glucose transport, exercise

3.1 Introduction

Aging is associated with the development of skeletal muscle insulin resistance [19][8]. Cardiac muscle is also insulin sensitive tissue but it is unknown whether the myocardium develops insulin resistance with advancing age.

Cardiac muscle normally derives between 60 and 70% of its energy metabolism from FFA oxidation, with glucose and lactate metabolism providing the rest [34]. However, the contribution of free fatty-acid oxidation to overall cardiac metabolism decreases with increasing age [1][16] and thus the importance of glucose oxidation and glycogenolysis increases in terms of energy provision [22]. While of quantitatively lesser importance than skeletal muscle for glucose disposal, age-related insulin resistance of the heart is nevertheless a serious concern in different pathological conditions during which glucose can become a more important metabolic fuel than fatty acids. Such states include acute energy deficits [12], chronic increase in workload such as hypertension [32], and myocardial ischemia where the heart must rely primarily on anaerobic glycolysis of exogenous glucose and endogenous glycogen [24]. Thus any process that limits exogenous glucose availability in these conditions, such as a compromised glucose transport system, can adversely affect myocardial function and survival.

Very little is known about whether the myocardial glucose transport system experiences age-related changes and develops insulin resistance similar to its skeletal muscle counterpart. Few studies have attempted to document changes in various components of the system during aging. Only myocardial Glut 4 content has been quantified, and in rats, it was reported to undergo a small age-related decrease [6][14]. If the myocardial glucose transport

system does behave like the skeletal muscle system, then the heart may become compromised in the face of a metabolic challenge.

Muscular contraction has been shown to cause recruitment of glucose transporters to the muscle plasma membrane and therefore increase glucose uptake [18][10][13]. This effect of contraction appears to be transduced by way of a signalling mechanism distinct from, and additive to, that of insulin [11] and appears to remain normal in insulin-resistant muscle [5].

For these reasons, physical exercise is an effective way to lower blood glucose in the presence of decreased insulin sensitivity and is now recognized as an important tool for the management of diabetes. It has been proposed that exercise can help prevent hyperglycemia and can thus halt the progression of pre-diabetic insulin resistance to full blown diabetes [3]. This has been supported by epidemiological studies suggesting that exercise reduces the risk for developing NIDDM [33][17][21].

Exercise has been shown to enhance the skeletal muscle glucose transport system. Insulin sensitivity is increased by both acute and chronic exercise, and this effect is persistent for up to 2 days after a single bout of acute exercise and up to 4 days following the last bout of chronic exercise [4]. Enhanced insulin-stimulated glucose transport has also been demonstrated in aged muscle [7]. Chronic activity has been shown to increase insulin-regulatable transporter content in a variety of skeletal muscle [28][27] while inactivity has been shown to decrease it [23]. Increase in Glut 4 transporter content of aged muscle also appears to be possible with exercise training [36]. Other components of the muscle glucose transport system have received less attention but insulin receptor number does appear to increase with exercise training [9][29].

Exercise also affects the myocardial glucose transport system. It has been shown that exercise training increases cardiac glucose uptake during rest and exercise [20], increases glycogen stores [30], and is cardioprotective against hypoxia [35]. It is well documented that the aged myocardium responds positively to exercise training, as shown by improved myocardial function and aerobic energy metabolism [31]. In addition, exercise training has been shown to attenuate the decrease in myocardial glucose transporter content observed in experimentally-induced diabetes [15]. This may explain how exercise training also partially normalizes myocardial glucose utilization depressed by diabetes [25]. However, it has been reported that exercise training is ineffective at attenuating the small age-related decrease in myocardial transporter content [14]. It is unknown if exercise can upregulate the content or function of other components of the myocardial glucose transport system.

The purpose of the present study was to investigate the effects of exercise, both chronic and acute, on several components of the aged myocardial glucose transport system, many of which have heretofore not been studied. Specifically, the content of Glut 4 and Glut 1 transporters, insulin receptor and IGF-1 receptor, and the signalling intermediate PI3 kinase were assessed by immunoblot.

3.2 Methods

Animals

Female C57Bl/6 mice were used for this study. Animals were obtained from the National Institute of Aging animal colony at 15 months of age and housed for nine months in conventional cages under laminar air flow. Animals were maintained on a 12:12 hr light-dark cycle and provided with

food (Purina Rodent Lab Diet 5001, Illinois, USA) and water ad libitum. Mice were randomly assigned to control (C), chronic exercise (CE), acute exercise (AE) and chronic + acute exercise (CAE) groups (n=6 animals per group). CE and CAE animals were individually housed in a cage containing a running wheel while C and AE animals were grouped housed (n=3-8). All experiments were performed at the same time of day following a 12 hour fast. Treatment of animals was certified by Simon Fraser University's animal care ethics committee, and all practices conformed to the regulations of the Canadian Council of Animal Care.

Exercise

Chronic exercise consisted of unlimited access to a 16 cm diameter running wheel. Voluntary wheel running was monitored by magnetic switches connected via an A-D board to a microcomputer. Total distance run was calculated and expressed per 24 hours. All animals in chronic exercise groups ran at least 1.2 km/day. Access to wheels was removed 48 hours prior to sacrifice. Acute exercise consisted of one forced bout of treadmill running on a motorized treadmill at 8 m/min up an 8% incline for a total duration of 2.5 hours. The animals were encouraged to run by jets of compressed air triggered by a light beam at the bottom of the treadmill. Experiments were conducted 12 hours after cessation of exercise.

Tissue preparation

Hearts were removed under sodium pentobarbital anesthesia (50 mg/kg), blotted, immediately frozen in liquid nitrogen and stored at -80°C until analysis. The inferior half of the heart composed entirely of ventricular tissue was removed, weighed and homogenized in 15 volumes of ice-cold

buffer containing 100 mM HEPES, pH 7.6; 150 mM NaCl; 5 mM EDTA; 5 mM $MgCl_2$; 2% Nonidet P-40; 1% Triton X-100; the following protease inhibitors: 2 mM phenylmethylsulfonyl fluoride (PMSF), 60 μM leupeptin, 60 μM pepstatin, 100 IU/ml bacitracin, 3 mg/ml aprotinin, 1 mg/ml benzamidine, 1 mg/ml N∝-p-tosyl-l-arginine methyl ester (TAME), 1 mg/ml N∝-benzoyl-l-arginine ethyl ester (BAEE); and the following phosphatase inhibitors: 100 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate. The homogenate was centrifuged at 10 000 g for 20 min at 4°C and the resulting supernatant was centrifuged at 150 000 g for 60 min at 4°C. The protein content of the final supernatant was determined by Bradford protein assay (Bio-Rad) using BSA standards. All samples were within the linear range of 2-12 μg. Crude solubilized membrane extracts were frozen at -80°C until further analysis.

Western blots

Crude membrane preparation containing 200 μg of protein was mixed with sample buffer containing 60 mM Tris, pH 6.8; 2% wt/vol SDS; 10% vol/vol glycerol, 5% vol/vol β-mercaptoethanol to a final volume of 125μl, boiled for 5 min and separated by SDS-PAGE on a 10% acrylamide 0.1% bis-acrylamide gel. Proteins were electrotransferred 3 hrs at 60V to 0.45μm PVDF membrane (Millipore), visualized by Ponceau Red (0.2% in 3% TCA, Sigma) and washed in TBST (50 mM Tris, pH 7.4; 150 mM NaCl; 0.5% Triton X-100). Membranes were blocked in TBST containing 3% BSA, washed in TBST and incubated overnight at 4°C with primary antibody. Membranes were washed in TBST, incubated with HRP-conjugated secondary antibody 90 min at 25°C and washed in TBST followed by TBS (50 mM Tris, pH 7.4; 150 mM NaCl). Membranes were immersed in Enhanced Chemi-Luminescence reagent (Amersham) and exposed to Hyperfilm (Amersham). Developed films were

quantified by laser densitometry using an LKB UltroScan XL densitometer and GelScan XL software. In order to allow comparisons across treatment groups, all samples were subjected to gel electrophoresis simultaneously, the gels were cut at appropriate molecular weight and transferred simultaneously to a single membrane.

Except where indicated, all antibodies were obtained from Santa Cruz Biotechnology. The following primary antibodies and concentrations were used for immunoblotting: goat-polyclonal anti-GLUT-4, 1:5000; rabbit-polyclonal anti-GLUT-1, 1:3333 (East-Acres Biologicals); mouse monoclonal anti-insulin receptor β-subunit, 1:10 000; rabbit polyclonal anti-IGF-1 β-subunit, 1:5000; rabbit polyclonal anti-p85 α-subunit, 1:5000. All primary antibodies were prepared in TBST, 1% BSA, and 0.5% sodium azide.

The following HRP-conjugated secondary antibodies and concentrations were used for immunoblotting: donkey anti-goat, 1:30 000; goat anti-rabbit, 1:30 000; goat anti-mouse, 1:30 000. All secondary antibodies were prepared fresh in TBST and 1% BSA.

Statistical Analysis

Statistical comparisons of western blot results between 4 treatment conditions were performed by one-way ANOVA for each dependent variable. Significance was set at the 0.05 level. Significant differences were analyzed post-hoc using a Duncan test. All statistical analysis was performed using SPSS 6.1 software for Macintosh.

3.3 Results

Descriptive data for the various treatment groups is contained in Table 3.1. No differences were observed in body weight or heart weight, however soleus weight was increased in the chronic wheel running conditions (CE and CAE). Specific protein levels are expressed relative to control group values and are outlined in Table 3.2 and illustrated in Figure 3.1 expressed per µg protein and in Figure 3.2 expressed per mg wet tissue. Protein yields remained relatively constant between treatment groups (Table 3.2).

Significant changes were observed in three of the five myocardial glucose transport system related proteins studied. Ventricular Glut 1 transporter and insulin receptor protein contents appear to be affected by acute exercise, either alone (AE) or in combination with chronic exercise (CAE). But chronic exercise alone (CE) does not alter levels of these two proteins. Glut 1 protein content showed an increased trend with acute exercise. The increase was only significant in the CAE group where it reached 114% above control levels (Figure 3.2B) when expressed per mg wet tissue, and 83% above control, when expressed per 200µg protein (Figure 3.1B). By contrast, insulin receptor protein content was decreased by acute exercise. Levels were reduced to 66% of control in the AE group and 56% of control in the CAE group (Figure 3.2C) when expressed per mg wet tissue, or 72% and 48% of control in the AE and CAE groups respectively (Figure 3.1C) when expressed per 200µg protein. Both differences were significant. Glut 4 transporter protein levels showed a similar decreasing trend as insulin receptor content with acute exercise, but decreases in AE and CAE groups did not reach significance (Figure 3.1A). PI3 kinase protein content was also affected by exercise, but was unchanged by either acute or chronic exercise alone. A combination of both types of exercise increased levels significantly in

the CAE group to 33% above control levels when expressed per mg wet tissue (Figure 3.2E). The difference fell short of significance when expressed per µg protein. Ventricular IGF-1 receptor content does not appear to be affected by exercise in a similar way to insulin receptors. Content was unaffected by any form of exercise (Figure 3.1D).

3.4 Discussion

The results of the present study have yielded a number of new observations. It appears that some components of the aged C57Bl/6 mouse myocardial glucose transport system are capable of responding to exercise. Long-term voluntary aerobic exercise was unable to elicit changes in protein content of any of the glucose transport related proteins studied here. However, it appears that the chronic activity may have had an effect at a level other than protein content, since a single 2.5 hour bout of acute aerobic exercise elicited larger changes in Glut 1 and PI3 kinase protein content in exercised animals than in control animals.

The inability of chronic exercise to induce change directly in the content of the proteins studied is in agreement with previous work demonstrating that 10 weeks of treadmill running cannot increase myocardial Glut 4 protein levels above age-matched sedentary levels in 25 month old rats [14]. We hypothesized that a longer duration stimulus might be necessary to cause changes in such a highly oxidative tissue as the heart and chose voluntary wheel running model of chronic exercise in order to simulate a lifetime of physical activity. However, due to the intermittent exercise pattern of the animals, this choice of model may have resulted in an exercise stimulus of a too low intensity for stimulating the heart. The mouse model was chosen for our study because it is a well established aging model.

Furthermore, the high intrinsic level of activity of the C57 mouse makes it a good candidate for voluntary exercise studies. In contrast to the myocardium, unpublished data from our lab show that this exercise and animal model was successful at promoting content changes in the skeletal muscle glucose transport system related proteins. The running velocity chosen for the acute exercise treatment was the same as the average velocity of chronic wheel running, however the exercise is more taxing as the animals run almost continuously for a total exercise time of 2.5 hours.

A parallel aging study from our laboratory using the same animal model suggests that the myocardial glucose transport system may be upregulated by advancing age (results reported in Chapter 2), in accordance to the well documented decrease in cardiac free fatty acid metabolism observed in aging [1][16] and the corresponding increase of glucose metabolism [22]. Furthermore, we observed that the heart weight and heart to body weight ratio of these animals undergo a significant age related increase not accompanied by a change in protein yield, suggesting cardiac hypertrophy. It is known that hypertrophy may also cause a decrease in the contribution of FFA metabolism to total cardiac energy production [2]. Such a change could also result in an upregulation of the glucose transport system. It is thus possible that changes due to chronic exercise would be overshadowed by age-induced and possibly hypertrophy-induced increases. It would be interesting to investigate the effects of a higher intensity chronic exercise model, however all chronic forced exercise studies in old animals are extremely labour intensive. Furthermore, because the heart is a highly oxidative tissue the effects of exercise are likely to result in changes of small magnitude requiring high statistical power to detect.

Glut 1 transporter content was markedly increased by acute exercise combined with previous nine month exposure to a running wheel, however the increase due to acute exercise alone was not statistically significant. This could be due to a "priming" of the system by chronic exercise whereby the magnitude of the effect of acute exercise is potentiated in the CAE group. The molecular basis of this effect could be a reduced degradation rate of Glut 1 protein or increased translational efficiency of Glut 1 mRNA with chronic exercise. A number of other events at the translational or transcriptional level could also be responsible, but the gene regulation of the transporter genes will need to be better understood before any hypotheses can be put forth. Another explanation would be that the effects of chronic exercise on Glut 1 protein content are short-lived and the protein levels have returned to control levels 48 hours after the last bout of chronic exercise. In this case, the acute exercise can be considered an extension of the chronic exercise regimen.

In contrast to Glut 1, Glut 4 transporter protein content was not shown to be altered by any form of exercise suggesting separate regulation of the two isoforms, in accordance to their different roles. As stated earlier, myocardial Glut 4 content has previously been shown to be unaffected by treadmill running in aged rats [14]. These findings imply that Glut 4 content is sufficient in the aged heart to meet the demands of contraction stimulated glucose transport. Findings from our aging study (Chapter 2) showed large age-related increases in Glut 4 content, and thus it is possible that aging-induced, and perhaps hypertrophy-induced, increases in Glut 4 have raised content to a level where no further increase can be elicited by exercise.

In contrast to the transporters, the ventricular insulin receptor protein content was decreased by acute exercise in both the AE and the CAE groups. Such a decrease would appear detrimental to insulin-stimulated glucose

transport. It is possible that this drop may be of a transient nature and be a response to the stress of the forced exercise bout. However measurements at multiple time points following activity would be necessary in order to assess this hypothesis. Alternatively, the decrease in insulin receptor content may be due to a shift away from insulin-stimulated glucose transport to contraction-stimulated transport, which operates through a pathway believed to be distinct from insulin [11][26]. Unlike the insulin receptor, the IGF-1 receptor content was not altered by any form of exercise. This is in agreement with skeletal muscle data from our laboratory.

We report elsewhere (Chapter 2) that myocardial PI3 kinase content varies with insulin and IGF-1 receptor content during aging. However our results here show an increase of PI3 kinase content in the CAE group which would suggest separate regulation of the elements of the signalling pathway in response to exercise. The fact that the PI3 kinase content increase is observable only with a combination of acute and chronic exercise, but not with either form of exercise alone, may also be due to a "priming" effect of chronic exercise, as discussed for Glut 1.

In conclusion, 9 months of chronic voluntary exercise was ineffective at eliciting changes directly at the protein content level, but may have potentiated the effects of acute exercise. Acute exercise, either alone or in combination with chronic exercise, was successful at eliciting changes in the content of some of the glucose transport related proteins of the aged myocardium, thus supporting the a priori hypothesis that insulin-independent components of the glucose transport system in the aged heart can be enhanced by exercise.

3.5 Acknowledgements

This work was supported by a grant from the British Columbia Health Research Foundation Council to W.S. Parkhouse and a fellowship from le Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR) to L.C. Martineau.

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3.7 Tables and figures

Table 3.1: Descriptive data for exercise groups

Exercise Condition	Sample Size (n=)	Heart Mass (mg)	Body Weight (g)	Heart / Body Weight Ratio	Soleus Mass (mg)
Control	6	128.9 ±7.6	32.7 ±3.7	.40%	7.4 ±0.6
Acute	6	124.7 ±12.1	28.4 ±2.1	.44%	7.8 ±0.9
Chronic	5	131.4 ±10.6	29.4 ±2.6	.45%	9.1 ±1.3
Acute + Chronic	5	131.5 ±12.5	28.1 ±2.5	.47%	8.9 ±0.9

Data expressed as mean ± SD

Table 3.2: Summary of western blot results

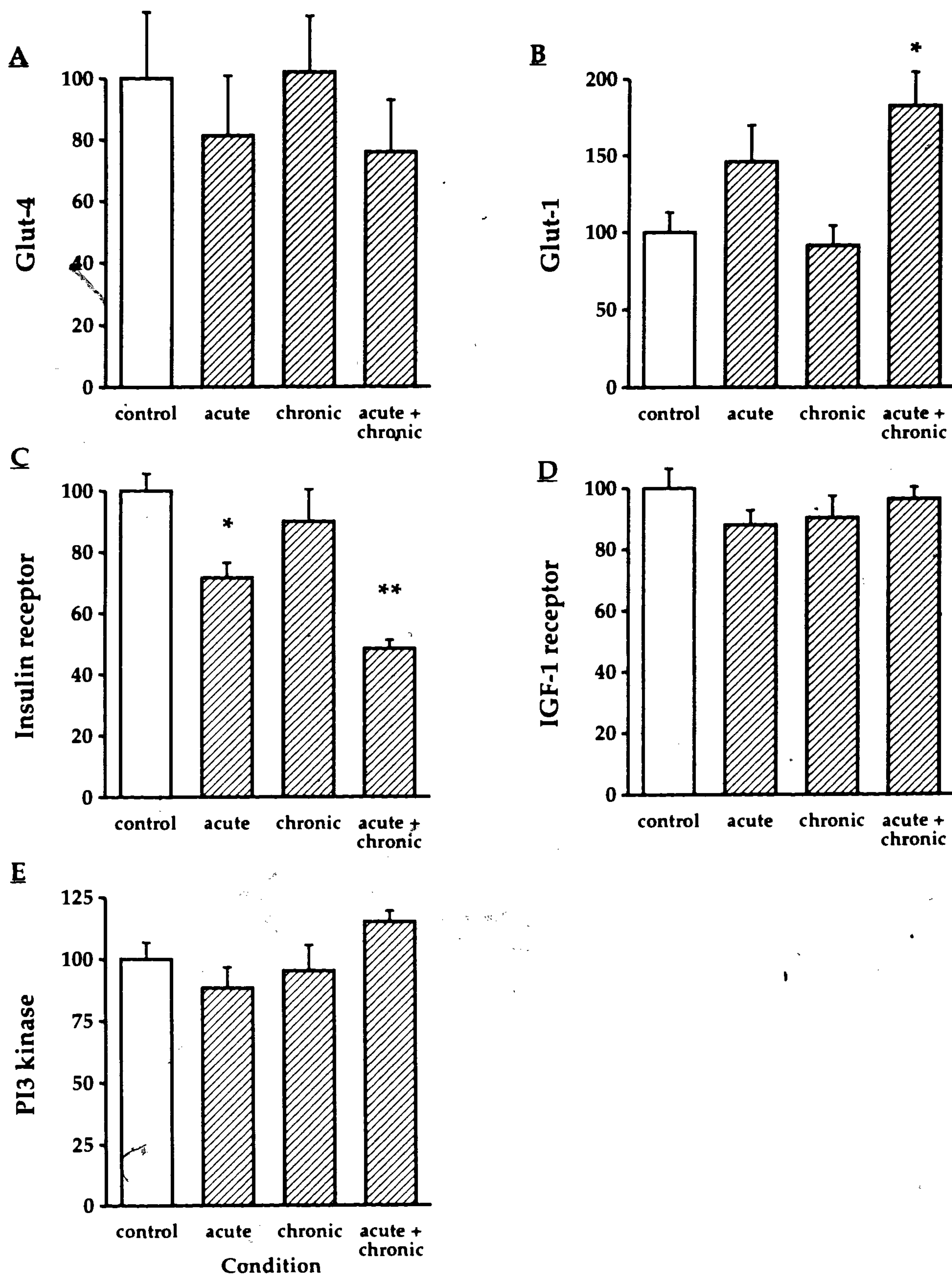
	Exercise Condition			
	Control	**Acute**	**Chronic**	**Acute + Chronic**
Crude protein yield	9.5% ±1.5	8.7% ±0.1	8.7% ±1.5	10.9% ±0.6
Glut-4				
% control/200μg protein	100 ±48.6	81.2 ±43.8	102.0 ±41.1	75.8 ±38.0
% control/mg wet tissue	100 ±44.5	80.3 ±43.2	103.4 ±46.8	94.2 ±47.4
Glut-1				
% control/200μg protein	100 ±31.6	145.9 ±58.3	91.5 ±28.7	182.6 ±49.5[a]
% control/mg wet tissue	100 ±32.1	135.3 ±53.6	87.5 ±37.9	214.1 ±66.7[b]
Insulin receptor				
% control/200μg protein	100 ±13.6	71.6 ±11.0[a]	89.8 ±23.6	48.3 ±6.2[b]
% control/mg wet tissue	100 ±16.2	66.4 ±10.4[a]	83.1 ±25.7	55.7 ±4.9[a]
IGF-1 receptor				
% control/200μg protein	100 ±15.7	88.0 ±11.6	90.3 ±15.8	96.4 ±8.7
% control/mg wet tissue	100 ±23.0	80.7 ±10.3	82.9 ±22.1	110.6 ±11.2
PI3 Kinase				
% control/200μg protein	100 ±16.7	88.4 ±20.2	95.3 ±23.3	115.0 ±9.9
% control/mg wet tissue	100 ±19.4	82.0 ±19.6	88.7 ±28.8	133.3 ±15.9[b]

protein data expressed as mean ± SD normalized as % of control group
n= 5-6 for all cells

a = different from control ($p<0.05$)
b = different from all other groups ($p<0.05$)

Table 3.2 Summary of western blot results for 5 proteins, expressed per 200 μg protein and per mg wet tissue

Figure 3.1: Graphical summary of western blot results expressed per 200 μg protein

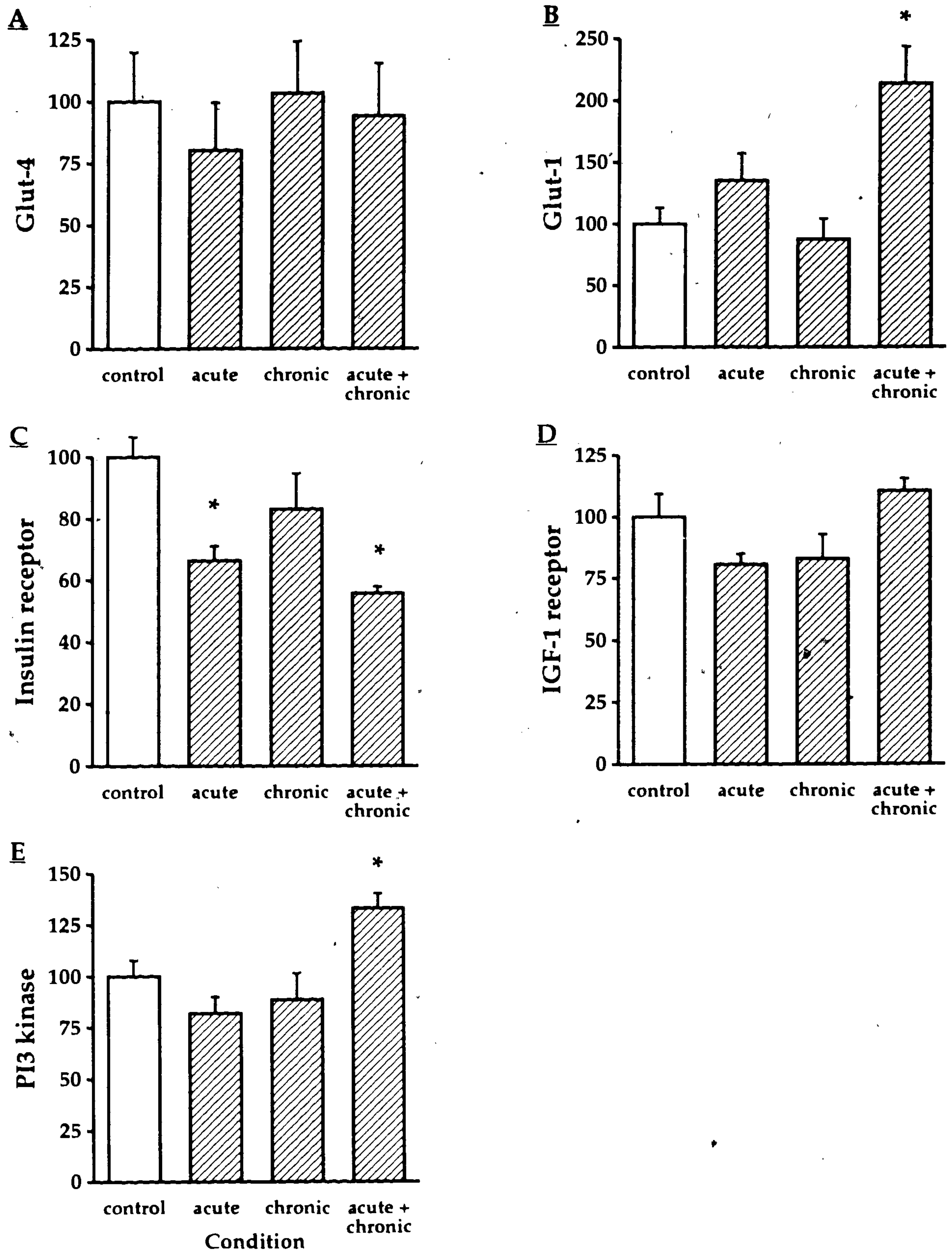


Data expressed as % of control group / 200 μg protein

* significantly different from control group ($p<0.05$)

** significantly different from all other groups ($p<0.05$)

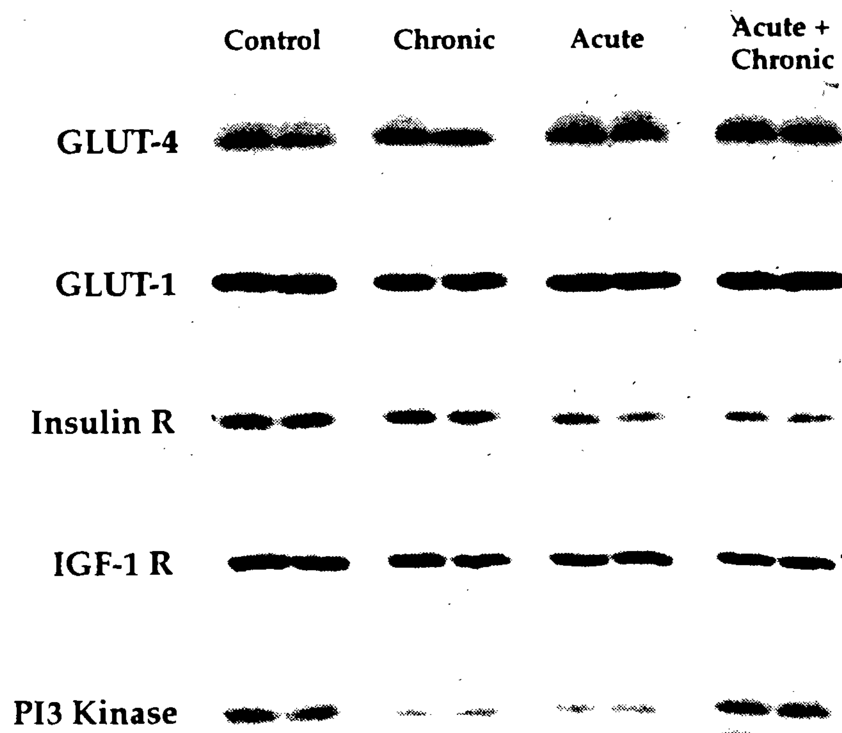
Figure 3.2: Graphical summary of western blot results expressed per mg wet tissue



Data expressed as % of control group / mg wet tissue

* significantly different from control group ($p<0.05$)

Figure 3.3: Representative immunoblots



CHAPTER 4
INTEGRATED CONCLUSION AND FUTURE DIRECTIONS

4.1 Integrated conclusion

The two related studies presented in this thesis were conceptualized together and carried out simultaneously. The original hypothesis at the root of both studies was that the aging process would cause a downregulation of the myocardial glucose transport system. Such a change could cause the myocardium to become compromised in certain pathological situations which require an increase in cardiac glucose metabolism [7][18][17]. This hypothesis was based on findings of skeletal muscle insulin resistance [12][6] and a downregulated glucose transport system [13][14][16][2][8][5][11][4][19] in aging skeletal muscle as well as limited reports of myocardial Glut 4 transporter content decreases with advancing age [3][9]. The aging study was designed to document any such downregulation at the protein level in cardiac muscle, while the exercise study was designed to evaluate whether the expected downregulation from the first study could be attenuated by various forms of exercise.

The unexpected results from the first study refuted the hypothesis of an age-related downregulation of the myocardial glucose transport system. It was in fact observed that this system undergoes an age-related upregulation at the protein level of some of its important components. These findings took away some impetus from the exercise study, as it could no longer be realistic to expect exercise-induced upregulation in an already enhanced system. However, findings from this study showed that exercise did produce an effect, although a limited and ambivalent one. Chronic exercise alone had no direct effect on protein content but appeared to potentiate the effects of acute

exercise, which in turn caused increased protein content of two components, while reducing a third. These effects were interpreted as a retained ability to partially upregulate the glucose transport system in the aged myocardium.

The significance of these surprising findings is questionable in light of the fact that functional measurements of glucose transport, the bottom line of the glucose transport system, were not made. Nevertheless, the findings of the aging study support the notion that the heart undergoes profound metabolic changes between maturity and old age. This is corroborated by the well documented decrease in cardiac free fatty acid metabolism observed in aging [1][10] and the corresponding increase of glucose metabolism [15]. The findings suggest enhanced transport capacity. This would imply that the heart may be less likely to develop insulin resistance than skeletal muscle and suggest that the aged myocardium may be less at risk for dealing with a metabolic challenge than previously hypothesized. The exercise data supports the practice of exercise as a cardioprotective measure.

4.2 Future directions

Because much of the work presented in this thesis is novel, further experiments will be needed in order to validate the results. Most importantly, validation of the physiological relevance of the findings must be demonstrated by assessing glucose transport, the only physiological measure of the functionality of the glucose transport system. Basal, insulin-stimulated, IGF-1-stimulated, contraction-stimulated, and hypoxia-stimulated glucose transport will all need to be measured in an aging study of the heart and in an exercise study of the aged myocardium in order to confirm the conclusions presented here. Further characterization of the C57Bl/6 mouse model, in terms of age and exercise-related changes in circulating insulin, IGF-1, glucose

and FFA levels, will be necessary in order to rule out inter-species differences as the main explanation for the divergence of our results from the rat literature and to establish the appropriateness of this model for the study of age-related insulin resistance. Replication of the work in a rat model may be useful and may eliminate the possible confounding effect of age-related cardiac hypertrophy observed in C57Bl/6 mice. This cardiac hypertrophy should also be further investigated histologically to confirm myocyte hypertrophy. In light of the small magnitude of the effects observed in the exercise study and the high degree of variability in measurements, the use of bigger sample sizes would be recommended in order to increase statistical power.

The present studies measured protein content and function of some essential components of the muscle glucose transport system, but were by no means exhaustive. Many other potential sites of regulation by age or exercise exist within the myocardial glucose transport system which merit some attention. This list includes receptor function, such as ligand binding kinetics and tyrosine kinase activity, content and phosphorylation (both tyrosine and serine) of the receptor substrate IRS-1, content and activity of the phosphatase SH-PTP2, PI3 kinase activity, and suspected steps involved in Glut 4 trafficking. Events at the translational and transcriptional level should also be explored.

As more becomes known about the signalling pathways used to induce Glut 4 translocation and stimulated glucose transport, new potential sites of regulation will also need to be investigated. Similarly, when the exact defect responsible for skeletal muscle insulin resistance is discovered, research into a corresponding defect in the heart, as well as the effects of exercise on this site, will be warranted.

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Appendix A

AGING STUDY RAW DATA

Contents:

Summary table
Summary figures of western blot results
Raw data spreadsheets
Photos of analyzed western blot films

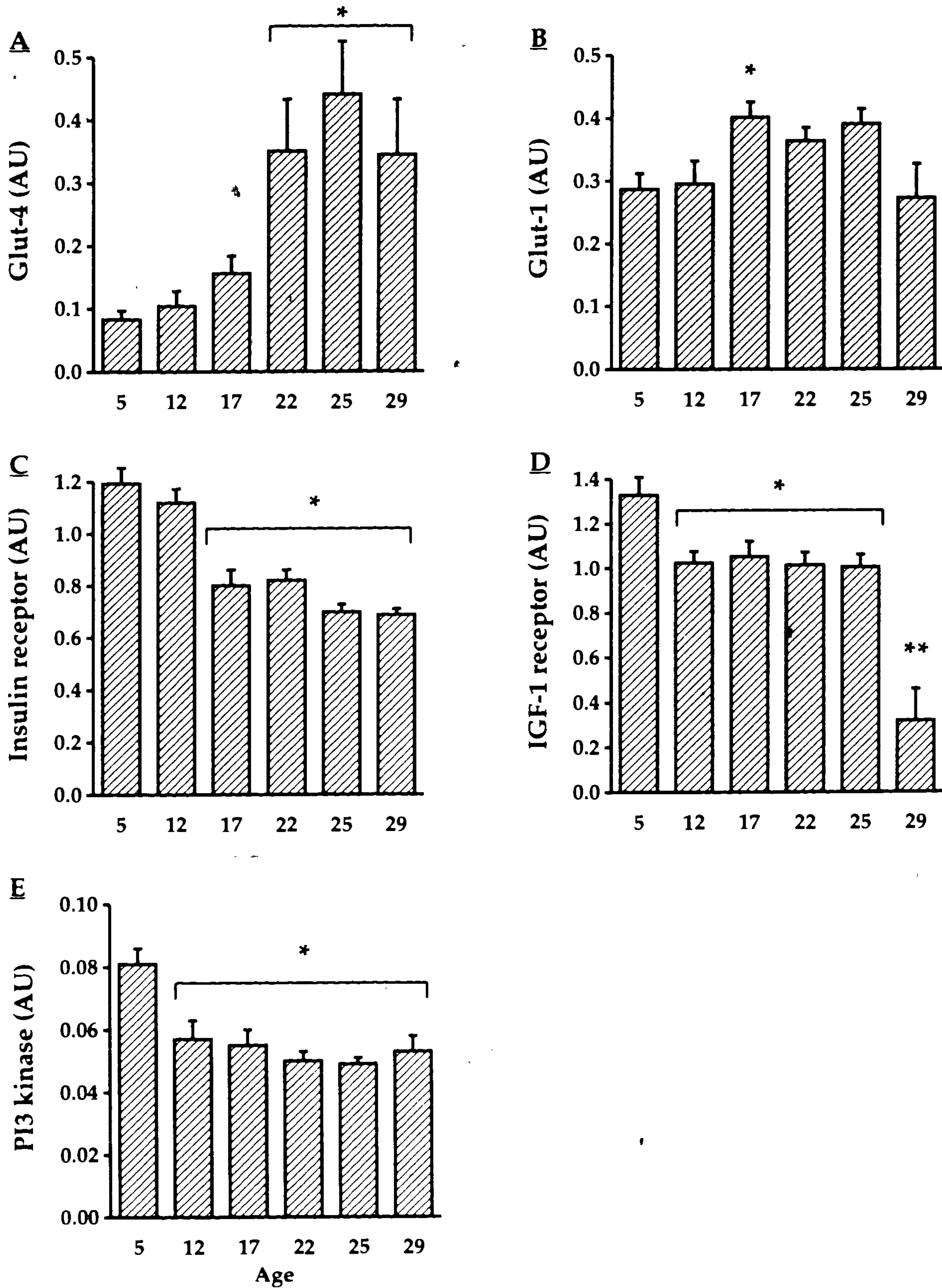
Appendix A: Summary of western blot results (in AU)

	Age (mo)					
	5	12	17	22.5	25	29
Crude protein yield	12.0% ±1.2	12.5% ±1.3	12.1% ±1.0	13.1% ±1.0	12.2% ±0.7	11.9% ±0.9
Glut-4						
AU/200µg protein	0.083 ±0.340	0.104 ±0.067	0.156 ±0.073	0.351 ±0.200[a]	0.441 ±0.223[a]	0.345 ±0.234[a]
AU/mg wet tissue	0.051 ±0.025	0.064 ±0.041	0.095 ±0.043	0.225 ±0.122[a]	0.269 ±0.129[a]	0.209 ±0.153[a]
	•		•	•	•	•
Glut-1						
AU/200µg protein	0.287 ±0.062	0.295 ±0.083	0.402 ±0.069[ab]	0.364 ±0.061	0.391 ±0.068[b]	0.273 ±0.154
AU/mg wet tissue	0.171 ±0.040	0.176 ±0.059	0.243 ±0.053[ab]	0.236 ±0.034[b]	0.239 ±0.042[ab]	0.161 ±0.086
	•	•				
Insulin Receptor						
AU/200µg protein	1.193 ±0.169	1.119 ±0.149	0.800 ±0.171[a]	0.820 ±0.114[a]	0.698 ±0.081[a]	0.687 ±0.064[a]
AU/mg wet tissue	0.707 ±0.048	0.694 ±0.035	0.476 ±0.069[ab]	0.533 ±0.060[ab]	0.425 ±0.037[a]	0.410 ±0.051[a]
IGF-1 Receptor						
AU/200µg protein	1.329 ±0.213	1.023 ±0.148[ab]	1.052 ±0.195[ab]	1.013 ±0.162[ab]	1.004 ±0.162[ab]	0.319 ±0.398[a]
AU/mg wet tissue	0.776 ±0.091	0.635 ±0.070[b]	0.626 ±0.076[b]	0.660 ±0.094[b]	0.613 ±0.100[b]	0.202 ±0.257[a]
	•					
PI3 Kinase						
AU/200µg protein	0.081 ±0.014	0.057 ±0.016[a]	0.055 ±0.015[a]	0.050 ±0.007[a]	0.049 ±0.007[a]	0.053 ±0.015[a]
AU/mg wet tissue	0.048 ±0.009	0.036 ±0.012[a]	0.033 ±0.007[a]	0.033 ±0.004[a]	0.030 ±0.004[a]	0.031 ±0.008[a]

protein data expressed as arbitrary units ± SD (AU defined as OD units x mm)
n=8 except where otherwise indicated by • (n=5-7)

a = different from 5 mo. control ($p<0.05$)
b = different from 29 mo. ($p<0.05$)

Appendix A: Graphical summary of western blot results expressed per 200 μg protein

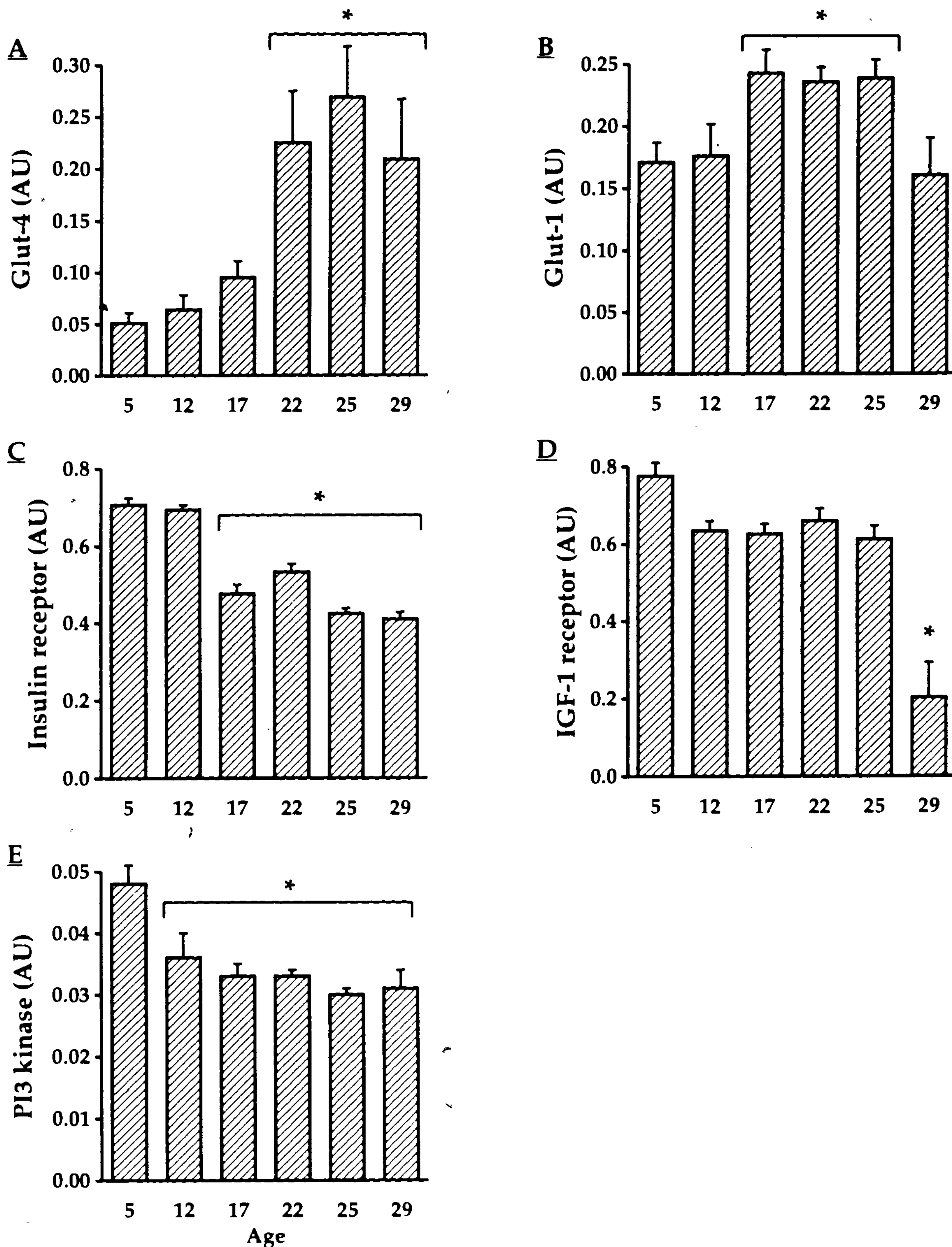


Data expressed as arbitrary units / 200 μg protein

* significantly different from 5 mo group ($p<0.05$)

** significantly different from all other groups ($p<0.05$)

Appendix A: Graphical summary of western blot results expressed per mg wet tissue



Data expressed as arbitrary units / mg wet tissue

* significantly different from 5 mo group ($p<0.05$)

Glut 4 western blot raw data

Band	5mo	12mo	17mo	22.5mo	25mo	29mo
1	discard	0.232	0.250	0.358	0.268	0.328
2	discard	0.241	0.294	0.699	0.269	0.449
3	discard	0.091	0.834	1.077	0.536	0.517
4	discard	0.081	0.734	0.928	0.502	0.474
5	0.077	0.136	0.087	0.431	1.240	0.241
6	0.040	0.180	0.078	0.335	1.094	0.205
7	0.047	0.044	0.272	1.439	0.404	0.118
8	0.049	0.030	0.169	1.548	0.341	0.140
9	0.100	0.079	0.179	0.570	0.719	1.359
10	0.112	0.096	0.213	0.536	0.671	1.456
11	0.062	0.041	0.128	0.459	0.689	0.212
12	0.039	0.041	0.105	0.405	0.763	0.210
13	0.148	0.082	0.101	0.113	0.399	0.188
14	0.097	0.043	0.080	0.079	0.405	0.174
15	0.186	0.157	0.142	0.139	0.129	0.958
16	0.041	0.083	0.084	0.091	0.084	0.620

Sample	5mo		12mo		17mo		22.5mo		25mo		29mo	
	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean
1	discard	**discard**	0.232	**0.237**	0.250	**0.272**	0.358	**0.529**	0.268	**0.269**	0.328	**0.389**
	discard		0.241		0.294		0.699		0.269		0.449	
2	discard	**discard**	0.091	**0.086**	outlier	**outlier**	outlier	**outlier**	0.536	**0.519**	0.517	**0.496**
	discard		0.081		outlier		outlier		0.502		0.474	
3	0.077	**0.059**	0.136	**0.158**	0.087	**0.083**	0.431	**0.383**	outlier	**outlier**	0.241	**0.223**
	0.040		0.180		0.078		0.335		outlier		0.205	
4	0.047	**0.048**	0.044	**0.037**	0.272	**0.221**	outlier	**outlier**	0.404	**0.373**	0.118	**0.129**
	0.049		0.030		0.169		outlier		0.341		0.140	
5	0.100	**0.106**	0.079	**0.088**	0.179	**0.196**	0.570	**0.553**	0.719	**0.695**	outlier	**outlier**
	0.112		0.096		0.213		0.536		0.671		outlier	
6	0.062	**0.051**	0.041	**0.041**	0.128	**0.117**	0.459	**0.432**	0.689	**0.726**	0.212	**0.211**
	0.039		0.041		0.105		0.405		0.763		0.210	
7	0.148	**0.123**	0.082	**0.063**	0.101	**0.091**	0.113	**0.096**	0.399	**0.402**	0.188	**0.181**
	0.097		0.043		0.080		0.079		0.405		0.174	
8	0.186	**0.114**	0.157	**0.120**	0.142	**0.113**	0.139	**0.115**	0.129	**0.107**	0.958	**0.789**
	0.041		0.083		0.084		0.091		0.084		0.620	
	Mean	**0.083**	**Mean**	**0.104**	**Mean**	**0.156**	**Mean**	**0.351**	**Mean**	**0.441**	**Mean**	**0.345**
	SD	0.034	**SD**	0.067	**SD**	0.073	**SD**	0.200	**SD**	0.223	**SD**	0.234
	SE	0.014	**SE**	0.024	**SE**	0.028	**SE**	0.082	**SE**	0.084	**SE**	0.088

Glut 1 western blot raw data

Band	5mo	12mo	17mo	22.5mo	25mo	29mo
1	discard	0.413	0.348	0.423	0.399	0.562
2	discard	0.418	0.218	0.387	0.510	0.609
3	discard	0.220	0.309	0.368	0.356	0.497
4	discard	0.143	0.354	0.279	0.269	0.398
5	0.196	0.312	0.400	0.358	0.275	0.308
6	0.160	0.182	0.336	0.332	0.377	0.306
7	0.388	0.090	0.486	0.415	0.433	0.163
8	0.334	0.107	0.476	0.365	0.423	0.163
9	0.295	0.064	0.444	0.425	0.394	0.201
10	0.262	0.105	0.505	0.455	0.430	0.176
11	0.365	0.077	0.490	0.453	0.396	0.144
12	0.304	0.088	0.374	0.407	0.403	0.135
13	0.271	0.213	0.406	0.303	0.484	0.183
14	0.204	0.215	0.443	0.285	0.493	0.191
15	0.332	0.273	0.461	0.254	0.319	0.166
16	0.296	0.354	0.376	0.311	0.299	0.211

Sample	5mo		12mo		17mo		22.5mo		25mo		29mo	
	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean
1	discard	**discard**	0.413	**0.416**	0.348	**0.283**	0.423	**0.405**	0.399	**0.455**	0.562	**0.562**
	discard		0.418		0.218		0.387		0.510		discard	
2	discard	**discard**	0.220	**0.220**	0.309	**0.332**	0.368	**0.324**	0.356	**0.313**	0.497	**0.448**
	discard		discard		0.354		0.279		0.269		0.398	
3	0.196	**0.196**	0.312	**0.312**	0.400	**0.368**	0.358	**0.345**	0.275	**0.326**	0.308	**0.307**
	discard		discard		0.336		0.332		0.377		0.306	
4	0.388	**0.361**	discard	**discard**	0.486	**0.481**	0.415	**0.390**	0.433	**0.428**	0.163	**0.163**
	0.334		discard		0.476		0.365		0.423		0.163	
5	0.295	**0.279**	discard	**discard**	0.444	**0.475**	0.425	**0.440**	0.394	**0.412**	0.201	**0.189**
	0.262		discard		0.505		0.455		0.430		0.176	
6	0.365	**0.335**	discard	**discard**	0.490	**0.432**	0.453	**0.430**	0.396	**0.400**	0.144	**0.140**
	0.304		discard		0.374		0.407		0.403		0.135	
7	0.271	**0.238**	0.213	**0.214**	0.406	**0.425**	0.303	**0.294**	0.484	**0.489**	0.183	**0.187**
	0.204		0.215		0.443		0.285		0.493		0.191	
8	0.332	**0.314**	0.273	**0.314**	0.461	**0.419**	0.254	**0.283**	0.319	**0.309**	0.166	**0.189**
	0.296		0.354		0.376		0.311		0.299		0.211	
	Mean	**0.287**	**Mean**	**0.295**	**Mean**	**0.402**	**Mean**	**0.364**	**Mean**	**0.391**	**Mean**	**0.273**
	SD	0.062	**SD**	0.083	**SD**	0.069	**SD**	0.061	**SD**	0.068	**SD**	0.154
	SE	0.025	**SE**	0.037	**SE**	0.024	**SE**	0.022	**SE**	0.024	**SE**	0.055

Insulin receptor western blot raw data

Band	5mo	12mo	17mo	22.5mo	25mo	29mo
1	1.091	1.114	0.909	0.997	0.810	0.692
2	1.054	1.099	0.805	0.784	0.686	0.658
3	1.322	1.583	1.144	1.004	0.690	0.655
4	1.294	1.344	1.105	0.927	0.551	0.595
5	1.400	0.987	0.747	0.849	0.724	0.697
6	1.327	1.169	0.863	0.883	0.606	0.768
7	1.334	1.014	0.836	0.956	0.633	0.782
8	1.422	0.975	0.793	0.790	0.539	0.633
9	1.324	1.073	0.662	0.914	0.678	0.646
10	1.210	1.080	0.591	0.833	0.620	0.512
11	1.202	0.977	0.881	0.752	0.898	0.662
12	1.241	1.013	0.702	0.806	0.722	0.685
13	0.962	1.267	0.935	0.719	0.838	0.811
14	0.914	1.045	0.737	0.656	0.741	0.751
15	1.087	1.203	0.642	0.685	0.784	0.816
16	0.911	0.966	0.452	0.569	0.644	0.627

Sample	5mo		12mo		17mo		22.5mo		25mo		29mo	
	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean
1	1.091	**1.073**	1.114	**1.107**	0.909	**0.857**	0.997	**0.891**	0.810	**0.748**	0.692	**0.675**
	1.054		1.099		0.805		0.784		0.686		0.658	
2	1.322	**1.308**	1.583	**1.464**	1.144	**1.125**	1.004	**0.966**	0.690	**0.621**	0.655	**0.625**
	1.294		1.344		1.105		0.927		0.551		0.595	
3	1.400	**1.364**	0.987	**1.078**	0.747	**0.805**	0.849	**0.866**	0.724	**0.665**	0.697	**0.733**
	1.327		1.169		0.863		0.883		0.606		0.768	
4	1.334	**1.378**	1.014	**0.995**	0.836	**0.815**	0.956	**0.873**	0.633	**0.586**	0.782	**0.708**
	1.422		0.975		0.793		0.790		0.539		0.633	
5	1.324	**1.267**	1.073	**1.077**	0.662	**0.627**	0.914	**0.874**	0.678	**0.649**	0.646	**0.579**
	1.210		1.080		0.591		0.833		0.620		0.512	
6	1.202	**1.222**	0.977	**0.995**	0.881	**0.792**	0.752	**0.779**	0.898	**0.810**	0.662	**0.674**
	1.241		1.013		0.702		0.806		0.722		0.685	
7	0.962	**0.938**	1.267	**1.156**	0.935	**0.836**	0.719	**0.688**	0.838	**0.790**	0.811	**0.781**
	0.914		1.045		0.737		0.656		0.741		0.751	
8	1.087	**0.999**	1.203	**1.085**	0.642	**0.547**	0.685	**0.627**	0.784	**0.714**	0.816	**0.722**
	0.911		0.966		0.452		0.569		0.644		0.627	
	Mean	**1.193**	**Mean**	**1.119**	**Mean**	**0.800**	**Mean**	**0.820**	**Mean**	**0.698**	**Mean**	**0.687**
	SD	0.169	**SD**	0.149	**SD**	0.171	**SD**	0.114	**SD**	0.081	**SD**	0.064
	SE	0.060	**SE**	0.053	**SE**	0.060	**SE**	0.040	**SE**	0.029	**SE**	0.023

IGF-1 receptor western blot raw data

Band	5mo	12mo	17mo	22.5mo	25mo	29mo
1	0.954	0.871	0.990	1.201	1.416	ND
2	0.938	0.985	1.148	1.290	1.053	ND
3	1.186	1.054	1.438	1.357	1.258	0.022
4	1.427	1.223	1.286	1.231	1.003	0.025
5	1.537	1.293	1.246	0.897	1.190	ND
6	1.591	1.281	1.254	0.934	1.177	ND
7	1.485	1.005	1.134	0.938	1.026	0.216
8	1.438	0.953	0.998	1.016	0.925	0.227
9	1.353	1.043	0.869	0.999	0.758	0.499
10	1.427	1.005	0.881	0.778	0.881	0.533
11	1.492	0.846	1.076	1.024	1.079	ND
12	1.455	0.725	1.108	0.924	0.906	ND
13	1.225	1.002	0.988	0.964	0.811	0.854
14	1.103	1.121	0.885	0.881	0.909	0.918
15	0.422	0.933	0.713	0.832	0.901	0.867
16	0.275	1.021	0.810	0.948	0.775	0.947

Sample	5mo		12mo		17mo		22.5mo		25mo		29mo	
	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean
1	0.954	**0.946**	0.871	**0.928**	0.990	**1.069**	1.201	**1.246**	1.416	**1.235**	ND	**0.000**
	0.938		0.985		1.148		1.290		1.053		ND	
2	1.186	**1.307**	1.054	**1.139**	1.438	**1.362**	1.357	**1.294**	1.258	**1.131**	0.022	**0.024**
	1.427		1.223		1.286		1.231		1.003		0.025	
3	1.537	**1.564**	1.293	**1.287**	1.246	**1.250**	0.897	**0.916**	1.190	**1.184**	ND	**0.000**
	1.591		1.281		1.254		0.934		1.177		ND	
4	1.485	**1.462**	1.005	**0.979**	1.134	**1.066**	0.938	**0.977**	1.026	**0.976**	0.216	**0.222**
	1.438		0.953		0.998		1.016		0.925		0.227	
5	1.353	**1.390**	1.043	**1.024**	0.869	**0.875**	0.999	**0.889**	0.758	**0.820**	0.499	**0.516**
	1.427		1.005		0.881		0.778		0.881		0.533	
6	1.492	**1.474**	0.846	**0.786**	1.076	**1.092**	1.024	**0.974**	1.079	**0.993**	ND	**0.000**
	1.455		0.725		1.108		0.924		0.906		ND	
7	1.225	**1.164**	1.002	**1.062**	0.988	**0.937**	0.964	**0.923**	0.811	**0.860**	0.854	**0.886**
	1.103		1.121		0.885		0.881		0.909		0.918	
8	outlier	**outlier**	0.933	**0.977**	0.713	**0.762**	0.832	**0.890**	0.901	**0.838**	0.867	**0.907**
	outlier		1.021		0.810		0.948		0.775		0.947	
	Mean	**1.329**	**Mean**	**1.023**	**Mean**	**1.052**	**Mean**	**1.013**	**Mean**	**1.004**	**Mean**	**0.319**
	SD	0.213	**SD**	0.148	**SD**	0.195	**SD**	0.162	**SD**	0.162	**SD**	0.398
	SE	0.080	**SE**	0.052	**SE**	0.069	**SE**	0.057	**SE**	0.057	**SE**	0.141

PI3 Kinase western blot raw data

Band	5mo	12mo	17mo	22.5mo	25mo	29mo
1	0.071	0.040	0.056	0.040	0.029	0.036
2	0.063	0.026	0.034	0.058	0.053	0.038
3	0.083	0.075	0.092	0.039	0.037	0.044
4	0.069	0.045	0.059	0.052	0.040	0.055
5	0.076	0.044	0.077	0.044	0.051	0.071
6	0.070	0.028	0.069	0.044	0.053	0.085
7	0.096	discard	0.074	0.060	0.056	0.059
8	0.119	0.054	0.051	0.031	0.050	0.070
9	0.072	0.077	0.053	0.055	0.056	0.058
10	0.064	0.054	0.038	0.059	0.041	0.062
11	0.089	0.088	discard	0.055	0.040	0.053
12	0.083	0.075	0.056	0.069	0.050	0.062
13	0.096	0.080	0.060	0.055	0.057	0.052
14	0.096	0.059	0.046	0.059	0.060	0.043
15	0.090	0.074	0.032	0.044	0.051	0.023
16	0.056	0.041	0.030	0.039	0.052	0.040

Sample	5mo		12mo		17mo		22.5mo		25mo		29mo	
	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean
1	0.071	**0.067**	0.040	**0.033**	0.056	**0.045**	0.040	**0.049**	0.029	**0.041**	0.036	**0.037**
	0.063		0.026		0.034		0.058		0.053		0.038	
2	0.083	**0.076**	0.075	**0.060**	0.092	**0.076**	0.039	**0.046**	0.037	**0.039**	0.044	**0.050**
	0.069		0.045		0.059		0.052		0.040		0.055	
3	0.076	**0.073**	0.044	**0.036**	0.077	**0.073**	0.044	**0.044**	0.051	**0.052**	0.071	**0.078**
	0.070		0.028		0.069		0.044		0.053		0.085	
4	0.096	**0.108**	discard	**0.054**	0.074	**0.063**	0.060	**0.046**	0.056	**0.053**	0.059	**0.065**
	0.119		0.054		0.051		0.031		0.050		0.070	
5	0.072	**0.068**	0.077	**0.066**	0.053	**0.046**	0.055	**0.057**	0.056	**0.049**	0.058	**0.060**
	0.064		0.054		0.038		0.059		0.041		0.062	
6	0.089	**0.086**	0.088	**0.082**	discard	**0.056**	0.055	**0.062**	0.040	**0.045**	0.053	**0.058**
	0.083		0.075		0.056		0.069		0.050		0.062	
7	0.096	**0.096**	0.080	**0.070**	0.060	**0.053**	0.055	**0.057**	0.057	**0.059**	0.052	**0.048**
	0.096		0.059		0.046		0.059		0.060		0.043	
8	0.090	**0.073**	0.074	**0.058**	0.032	**0.031**	0.044	**0.042**	0.051	**0.052**	0.023	**0.032**
	0.056		0.041		0.030		0.039		0.052		0.040	
	Mean	**0.081**	**Mean**	**0.057**	**Mean**	**0.055**	**Mean**	**0.050**	**Mean**	**0.049**	**Mean**	**0.053**
	SD	0.014	**SD**	0.016	**SD**	0.015	**SD**	0.007	**SD**	0.007	**SD**	0.015
	SE	0.005	**SE**	0.006	**SE**	0.005	**SE**	0.003	**SE**	0.002	**SE**	0.005

Insulin receptor autophosphorylation raw data

12 months

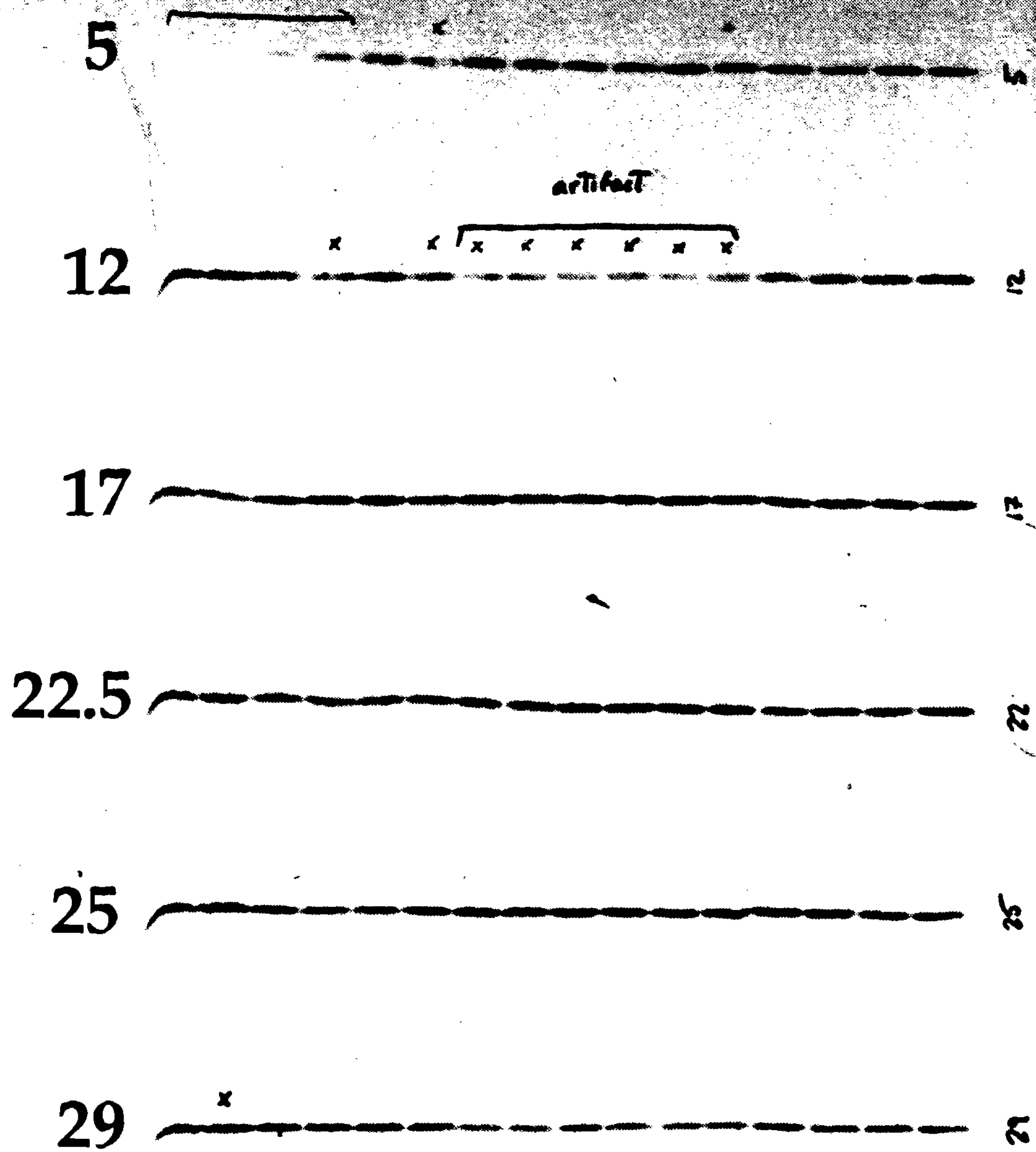
		Correction factor 1.119
Sample 1	Basal	0.163
	Maximal	0.559
	% increase	**242.9**
	Corrected Basal	0.146
	Corrected Max	0.500
Sample 2	Basal	0.283
	Maximal	0.825
	% increase	**191.5**
	Corrected Basal	0.253
	Corrected Max	0.737
Sample 3	Basal	0.201
	Maximal	0.492
	% increase	**144.8**
	Corrected Basal	0.180
	Corrected Max	0.440
Sample 4	Basal	0.096
	Maximal	0.167
	% increase	**74.0**
	Corrected Basal	0.086
	Corrected Max	0.149
Sample 5	Basal	0.123
	Maximal	0.384
	% increase	**212.2**
	Corrected Basal	0.110
	Corrected Max	0.343
Sample 6	Basal	0.291
	Maximal	0.684
	% increase	**135.1**
	Corrected Basal	0.260
	Corrected Max	0.611
	Mean % increase	**166.7**
	SD	61.0
	SE	24.9
	Mean Basal	**0.193**
	SD	0.081
	SE	0.033
	Mean maximum	**0.519**
	SD	0.287
	SE	0.117
	Mean corrected basal	**0.172**
	SD	0.073
	SE	0.030
	Mean corrected maximum	**0.463**
	SD	0.257
	SE	0.105

29 months

		Correction factor 0.687
Sample 1	Basal	0.115
	Maximal	0.347
	% increase	**201.7**
	Corrected Basal	0.167
	Corrected Max	0.505
Sample 2	Basal	0.196
	Maximal	0.387
	% increase	**97.4**
	Corrected Basal	0.285
	Corrected Max	0.563
Sample 3	Basal	0.182
	Maximal	0.571
	% increase	**213.7**
	Corrected Basal	0.265
	Corrected Max	0.831
Sample 4	Basal	0.145
	Maximal	0.704
	% increase	**385.5**
	Corrected Basal	0.211
	Corrected Max	1.025
Sample 5	Basal	0.374
	Maximal	1.108
	% increase	**196.3**
	Corrected Basal	0.544
	Corrected Max	1.613
Sample 6	Basal	0.352
	Maximal	1.150
	% increase	**226.7**
	Corrected Basal	0.512
	Corrected Max	1.674
	Mean % increase	**220.2**
	SD	93.2
	SE	38.0
	Mean Basal	**0.227**
	SD	0.109
	SE	0.045
	Mean maximum	**0.711**
	SD	0.417
	SE	0.170
	Mean corrected basal	**0.331**
	SD	0.159
	SE	0.065
	Mean corrected maximum	**1.035**
	SD	0.606
	SE	0.248

IGF-1 receptor autophosphorylation raw data

12 months		Correction factor 1.023		29 months		Correction factor 0.319
Sample 1	Basal	0.298	0.291	Sample 1	Basal	0.301
	Maximal	0.360			Maximal	0.389
	% increase	**20.8**			% increase	**29.2**
	Corrected Basal	0.291			Corrected Basal	0.944
	Corrected Max	0.352			Corrected Max	1.219
Sample 2	Basal	0.361	0.353	Sample 2	Basal	0.243
	Maximal	0.450			Maximal	0.452
	% increase	**24.7**			% increase	**86.0**
	Corrected Basal	0.353			Corrected Basal	0.762
	Corrected Max	0.440			Corrected Max	1.417
Sample 3	Basal	0.253	0.247	Sample 3	Basal	0.184
	Maximal	0.602			Maximal	0.212
	% increase	**137.9**			% increase	**15.2**
	Corrected Basal	0.247			Corrected Basal	0.577
	Corrected Max	0.588			Corrected Max	0.665
Sample 4	Basal	0.104	0.102	Sample 4	Basal	0.226
	Maximal	0.203			Maximal	0.392
	% increase	**95.2**			% increase	**73.5**
	Corrected Basal	0.102			Corrected Basal	0.708
	Corrected Max	0.198			Corrected Max	1.229
Sample 5	Basal	0.212	0.207	Sample 5	Basal	0.706
	Maximal	0.331			Maximal	0.710
	% increase	**56.1**			% increase	**0.6**
	Corrected Basal	0.207			Corrected Basal	2.213
	Corrected Max	0.324			Corrected Max	2.226
Sample 6	Basal	0.138	0.135	Sample 6	Basal	0.193
	Maximal	0.355			Maximal	0.207
	% increase	**157.2**			% increase	**7.3**
	Corrected Basal	0.135			Corrected Basal	0.605
	Corrected Max	0.347			Corrected Max	0.649
	Mean % increase	**82.0**			**Mean % increase**	**35.3**
	SD	57.7			SD	35.9
	SE	23.6			SE	14.7
	Mean Basal	**0.228**			**Mean Basal**	**0.309**
	SD	0.097			SD	0.199
	SE	0.040			SE	0.081
	Mean maximum	**0.384**			**Mean maximum**	**0.394**
	SD	0.189			SD	0.225
	SE	0.077			SE	0.092
	Mean corrected basal	**0.223**			**Mean corrected basal**	**0.968**
	SD	0.095			SD	0.624
	SE	0.039			SE	0.255
	Mean corrected maximum	**0.375**			**Mean corrected maximum**	**1.234**
	SD	0.185			SD	0.706
	SE	0.076			SE	0.288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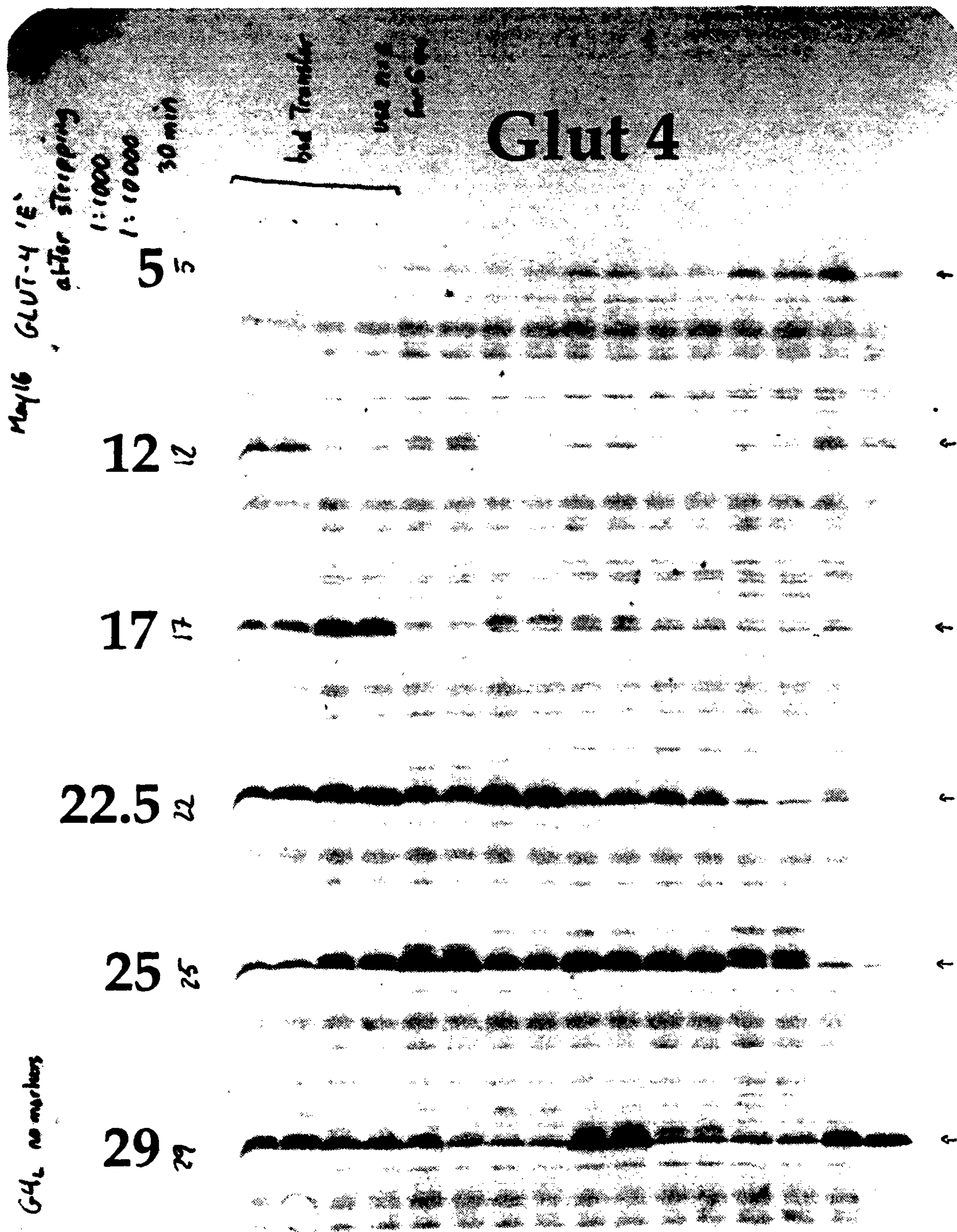
12
17
22.5
25
29
artifact
March 97
GLUT-1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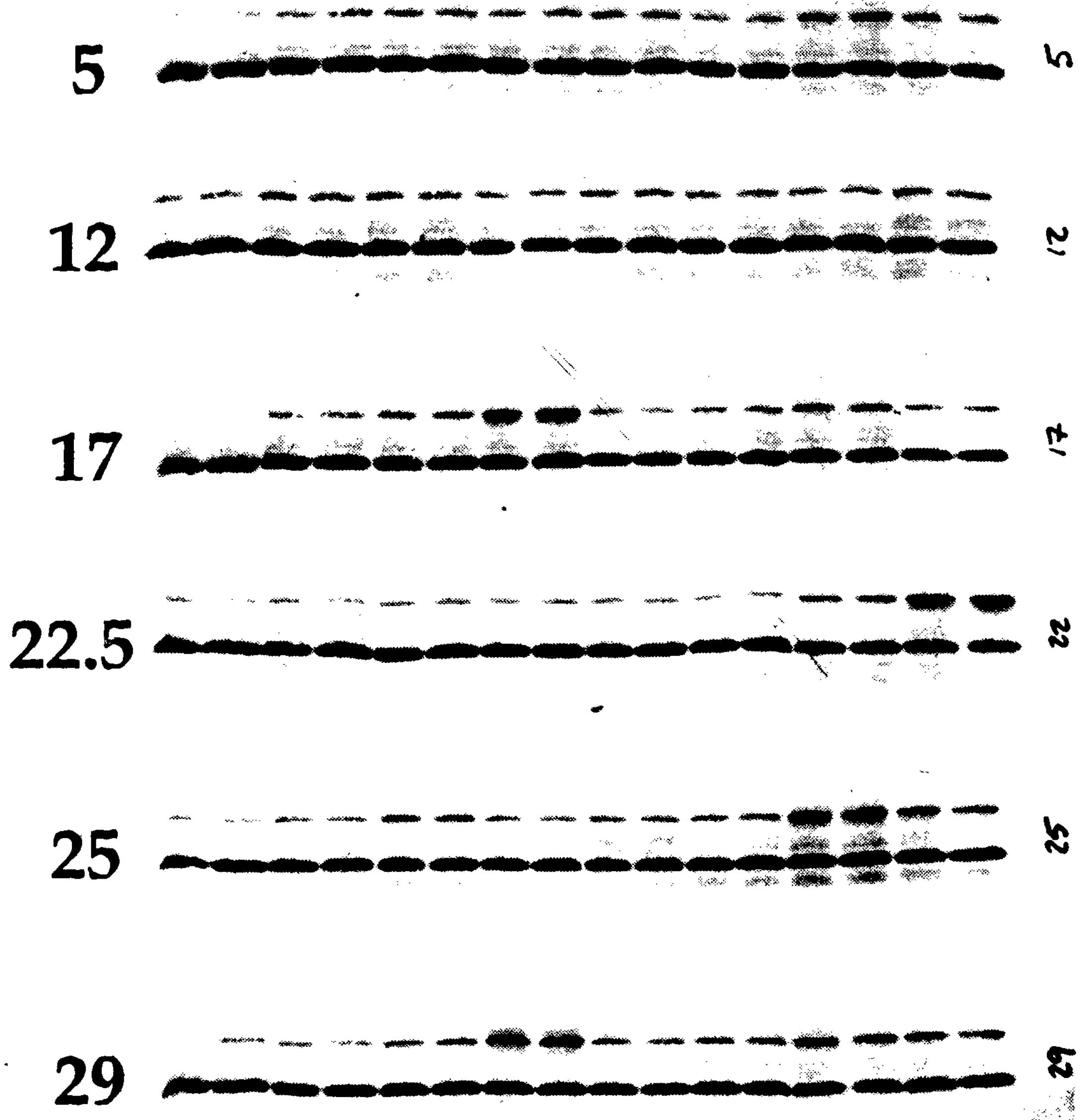
5
12
17
22.5
25
29


anti insulin R
October 96
1:5000
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Insulin R β
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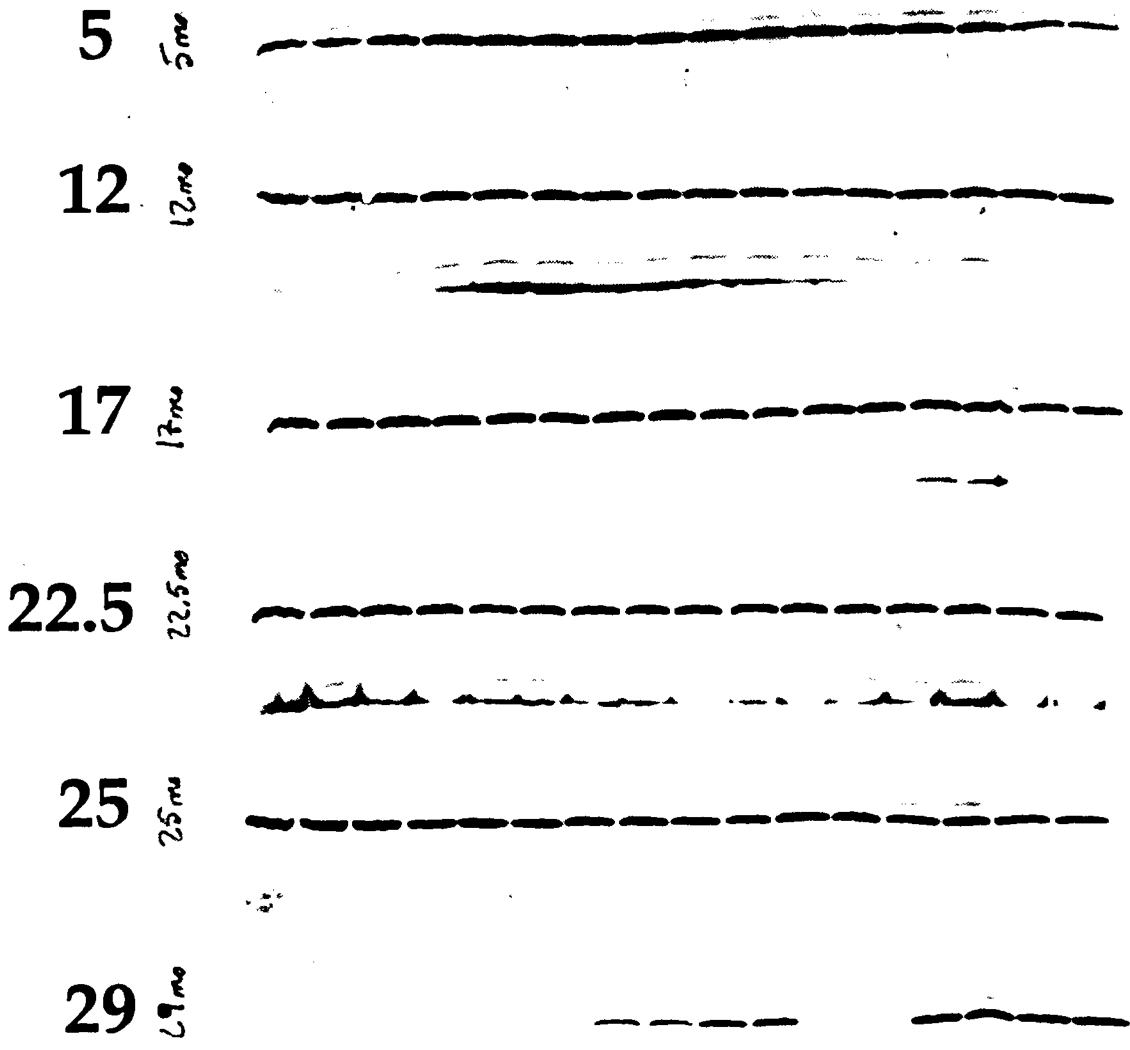
IGF-1 R β



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22.5

25

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Appendix B

EXERCISE STUDY RAW DATA

Contents: Summary table
Summary figures of western blot results
Raw data spreadsheets
Photos of analyzed western blot films

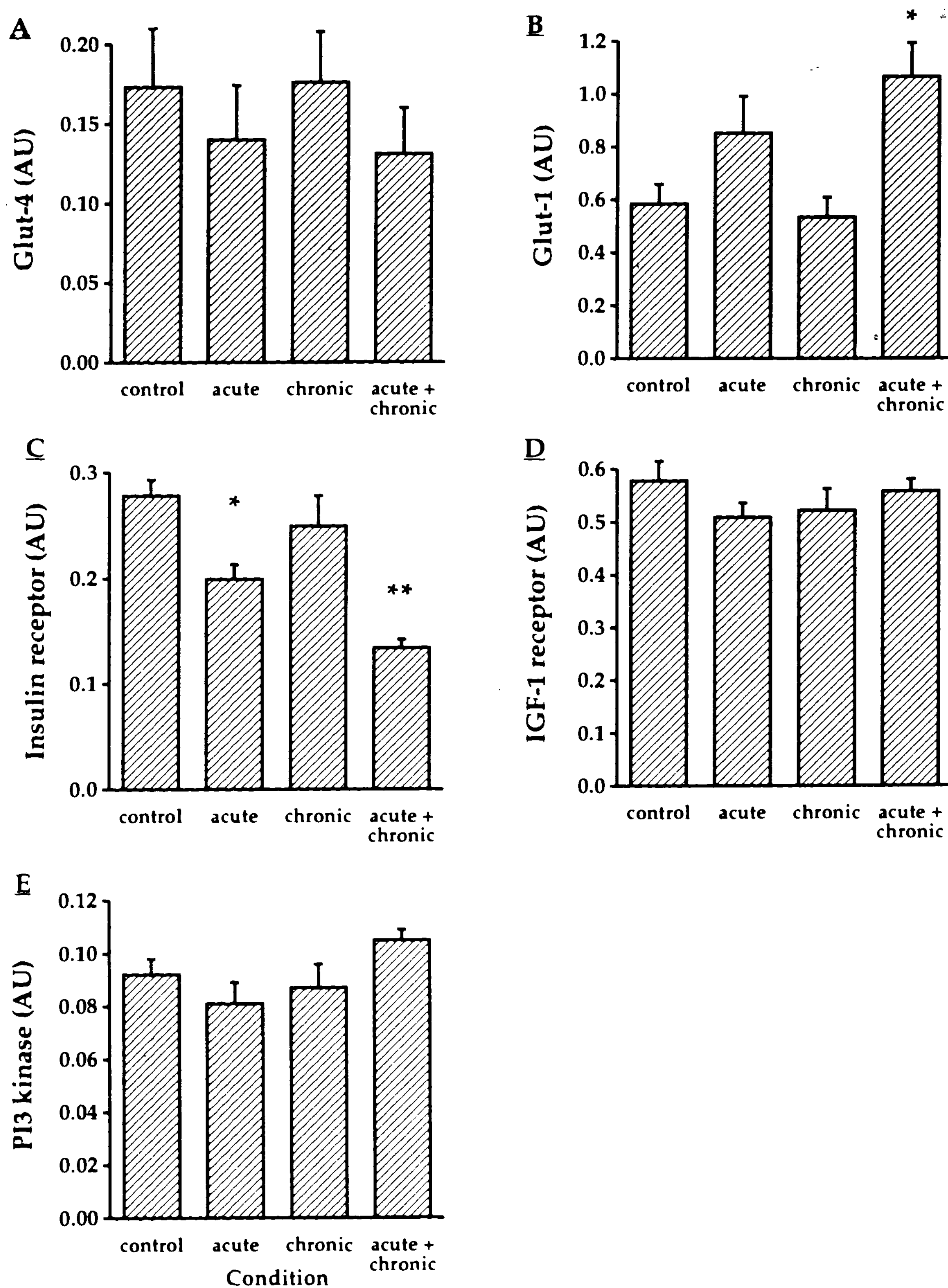
Appendix B: Summary of western blot results (in AU)

	Exercise Condition			
	Control	Acute	Chronic	Acute + Chronic
Crude protein yield	9.5% ±1.5	8.7% ±0.1	8.7% ±1.5	10.9% ±0.6
Glut-4				
AU/200μg protein	0.173 ±0.084	0.140 ±0.076	0.176 ±0.071	0.131 ±0.066
AU/mg wet tissue	0.076 ±0.034	0.061 ±0.033	0.079 ±0.036	0.072 ±0.036
Glut-1				
AU/200μg protein	0.584 ±0.184	0.852 ±0.340	0.534 ±0.168	1.066 ±0.289[a]
AU/mg wet tissue	0.274 ±0.088	0.371 ±0.147	0.240 ±0.104	0.587 ±0.183[b]
Insulin receptor				
AU/200μg protein	0.278 ±0.038	0.199 ±0.031[a]	0.249 ±0.065	0.134 ±0.017[b]
AU/mg wet tissue	0.131 ±0.021	0.087 ±0.014[a]	0.109 ±0.034	0.073 ±0.006[a]
IGF-1 receptor				
AU/200μg protein	0.578 ±0.091	0.509 ±0.067	0.522 ±0.091	0.558 ±0.051
AU/mg wet tissue	0.275 ±0.063	0.222 ±0.028	0.228 ±0.061	0.304 ±0.031
PI3 Kinase				
AU/200μg protein	0.092 ±0.015	0.081 ±0.019	0.087 ±0.021	0.105 ±0.009
AU/mg wet tissue	0.043 ±0.008	0.035 ±0.008	0.038 ±0.012	0.058 ±0.007[b]

protein data expressed as arbitrary units ± SD (AU defined as OD units x mm)
n= 5-6 for all cells

a = different from control ($p<0.05$)
b = different from all other groups ($p<0.05$)

Appendix B: Graphical summary of western blot results expressed per 200 μg protein

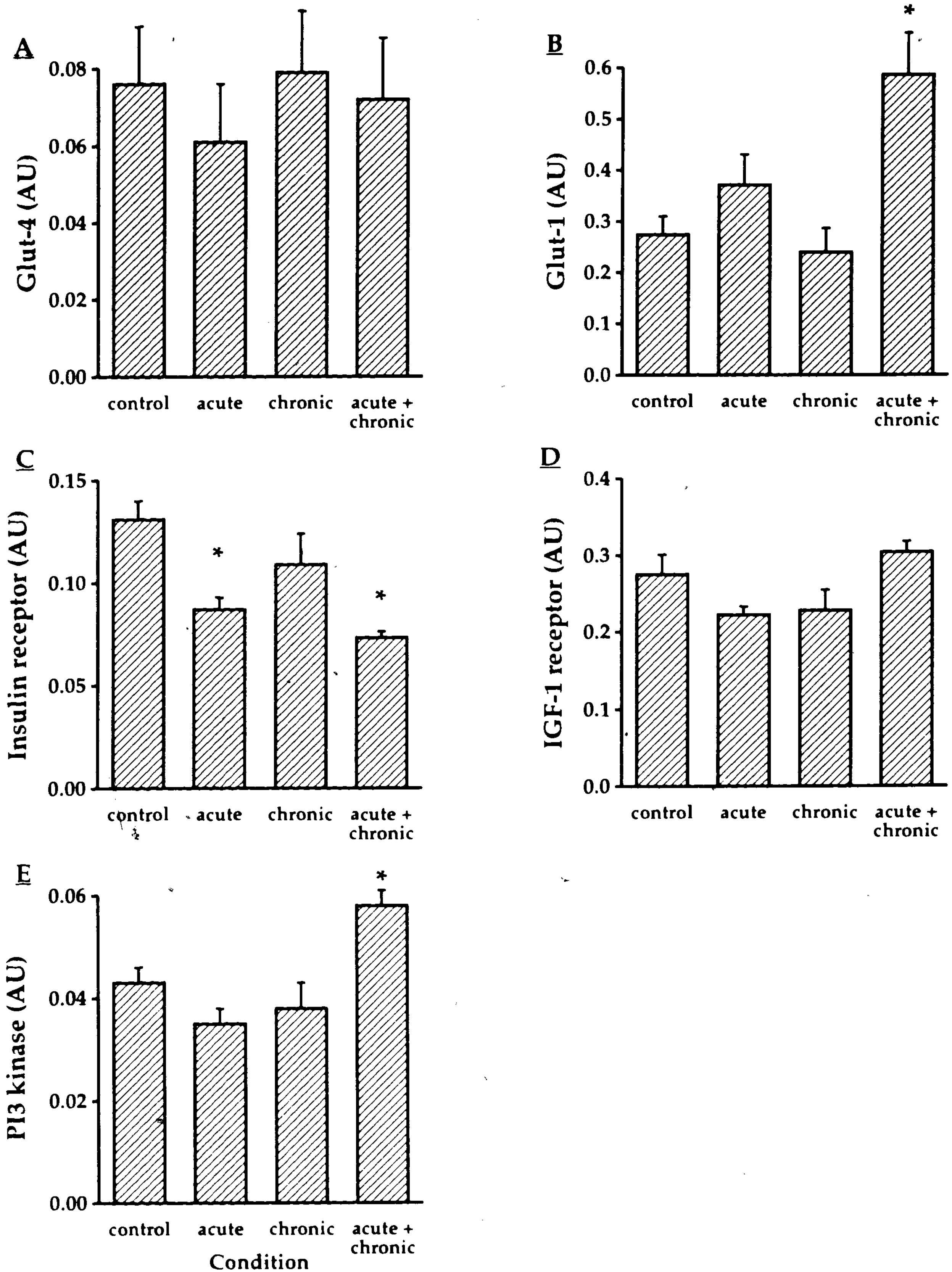


Data expressed in arbitrary units / 200 μg protein

* significantly different from control group ($p<0.05$)

** significantly different from all other groups ($p<0.05$)

Appendix B: Graphical summary of western blot results expressed per mg wet tissue



Data expressed as arbitrary units / mg wet tissue

* significantly different from control group ($p<0.05$)

Glut 4 western blot raw data

Band	Control	Chronic	Acute	A+C
1	0.275	0.132	0.066	0.042
2	0.321	0.166	0.080	0.045
3	0.863	0.063	0.054	0.219
4	0.939	0.073	0.070	0.154
5	0.162	0.214	0.830	0.080
6	0.112	0.171	0.855	0.100
7	0.073	0.259	0.166	discard
8	0.123	0.241	0.172	0.200
9	0.112	0.233	0.234	0.117
10	0.113	0.208	0.259	0.151
11	0.252		0.150	
12	0.183		0.069	

Sample	Control		Chronic		Acute		Acute + Chronic	
	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean
1	0.275	**0.298**	0.132	**0.149**	0.066	**0.073**	0.042	**0.044**
	0.321		0.166		0.080		0.045	
2	outlier	**outlier**	0.063	**0.068**	0.054	**0.062**	0.219	**0.187**
	outlier		0.073		0.070		0.154	
3	0.162	**0.137**	0.214	**0.193**	outlier	**outlier**	0.080	**0.090**
	0.112		0.171		outlier		0.100	
4	0.073	**0.098**	0.259	**0.250**	0.166	**0.169**	discard	**0.200**
	0.123		0.241		0.172		0.200	
5	0.112	**0.113**	0.233	**0.221**	0.234	**0.247**	0.117	**0.134**
	0.113		0.208		0.259		0.151	
6	0.252	**0.218**			0.150	**0.150**		
	0.183				discard			
	Mean	**0.173**	**Mean**	**0.176**	**Mean**	**0.140**	**Mean**	**0.131**
	SD	0.084	**SD**	0.071	**SD**	0.076	**SD**	0.066
	SE	0.038	**SE**	0.032	**SE**	0.034	**SE**	0.029

Glut 1 western blot raw data

Band	Control	Chronic	Acute	A+C
1	0.427	0.500	0.535	0.734
2	0.730	0.244	0.389	0.648
3	0.396	0.288	0.399	0.777
4	0.495	0.206	0.235	0.936
5	0.734	0.373	0.317	1.230
6	0.483	0.595	0.913	1.372
7	0.313	0.499	1.013	1.081
8	0.214	0.574	1.127	1.140
9	0.663	0.787	1.113	1.266
10	0.949	0.714	0.995	1.472
11	0.962		1.055	
12	0.538		1.369	

Sample	Control		Chronic		Acute		Acute + Chronic	
	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean
1	0.427	**0.579**	0.500	**0.500**	0.535	**0.462**	0.734	**0.691**
	0.730		discard		0.389		0.648	
2	0.396	**0.446**	0.288	**0.288**	0.399	**0.399**	0.777	**0.857**
	0.495		discard		discard		0.936	
3	0.734	**0.609**	discard	**0.595**	discard	**0.913**	1.230	**1.301**
	0.483		0.595		0.913		1.372	
4	0.313	**0.313**	0.499	**0.537**	1.013	**1.070**	1.081	**1.111**
	discard		0.574		1.127		1.140	
5	0.663	**0.806**	0.787	**0.751**	1.113	**1.054**	1.266	**1.369**
	0.949		0.714		0.995		1.472	
6	0.962	**0.750**			1.055	**1.212**		
	0.538				1.369			
	Mean	**0.584**	**Mean**	**0.534**	**Mean**	**0.852**	**Mean**	**1.066**
	SD	0.184	**SD**	0.168	**SD**	0.340	**SD**	0.289
	SE	0.075	**SE**	0.075	**SE**	0.139	**SE**	0.129

Insulin receptor western blot raw data

Band	Control	Chronic	Acute	A + C
1	0.315	0.199	0.179	0.166
2	0.318	0.206	0.181	0.137
3	0.297	0.163	0.184	0.155
4	0.244	0.215	0.201	0.141
5	0.309	0.273	0.389	0.156
6	0.187	0.262	0.376	0.119
7	0.115	0.389	0.223	0.122
8	0.219	0.317	0.231	0.099
9	0.317	0.258	0.233	0.122
10	0.282	0.212	0.157	0.124
11	0.292		0.194	
12	0.210		0.129	

Sample	Control		Chronic		Acute		Acute + Chronic	
	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean
1	0.315	**0.317**	0.199	**0.203**	0.179	**0.180**	0.166	**0.152**
	0.318		0.206		0.181		0.137	
2	0.297	**0.271**	0.163	**0.189**	0.184	**0.193**	0.155	**0.148**
	0.244		0.215		0.201		0.141	
3	0.309	**0.309**	0.273	**0.268**	outlier	**outlier**	0.156	**0.138**
	discard		0.262		outlier		0.119	
4	discard	**0.219**	0.389	**0.353**	0.223	**0.227**	0.122	**0.111**
	0.219		0.317		0.231		0.099	
5	0.317	**0.300**	0.258	**0.235**	0.233	**0.233**	0.122	**0.123**
	0.282		0.212		discard		0.124	
6	0.292	**0.251**			0.194	**0.162**		
	0.210				0.129			
	Mean	**0.278**	**Mean**	**0.249**	**Mean**	**0.199**	**Mean**	**0.134**
	SD	0.038	**SD**	0.065	**SD**	0.031	**SD**	0.017
	SE	0.015	**SE**	0.029	**SE**	0.014	**SE**	0.008

IGF-1 receptor western blot raw data

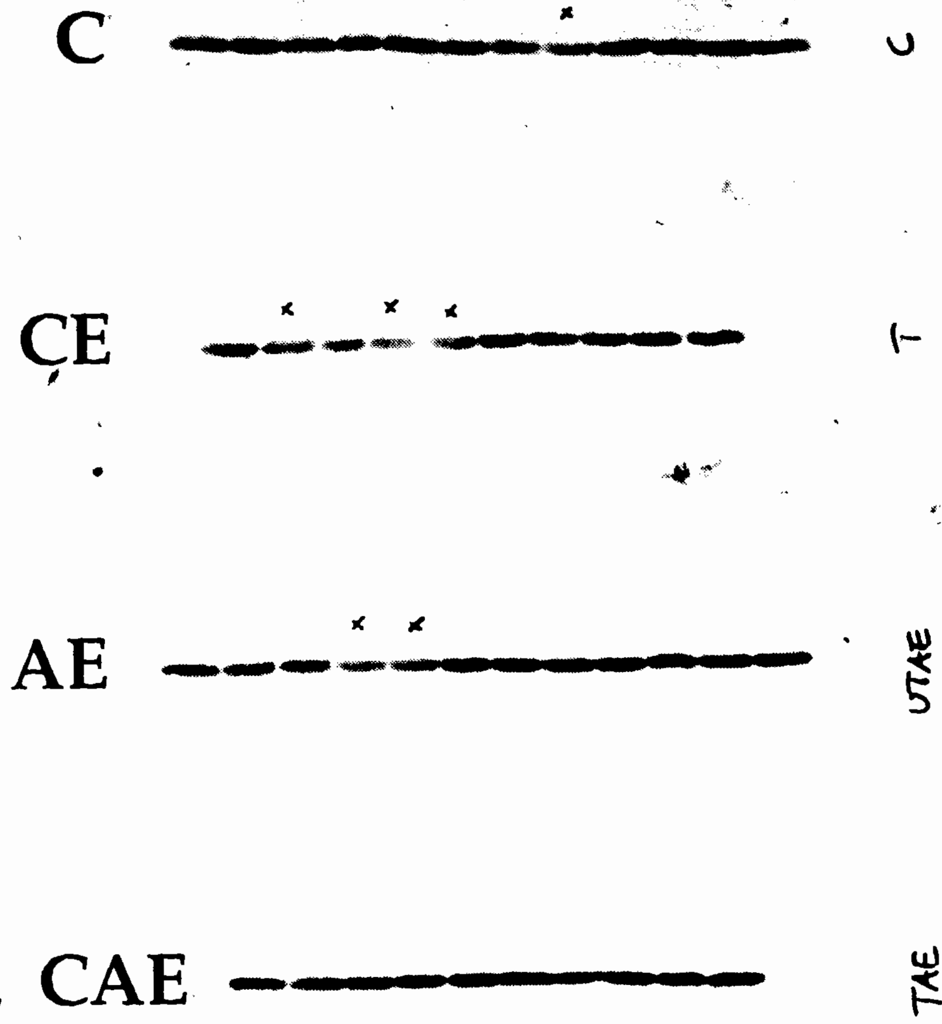
Band	Control	Chronic	Acute	A+C
1	0.650	0.635	0.438	0.615
2	0.597	0.591	0.403	0.473
3	0.484	0.430	0.410	0.542
4	0.667	0.370	0.501	0.527
5	0.551	0.492	0.513	0.713
6	0.399	0.416	0.577	0.553
7	0.357	0.626	0.428	0.648
8	0.586	0.482	0.585	0.507
9	0.536	0.622	0.553	0.568
10	0.716	0.557	0.671	0.430
11	0.709		0.511	
12	0.689		0.520	

Sample	Control		Chronic		Acute		Acute + Chronic	
	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean
1	0.650	**0.624**	0.635	**0.613**	0.438	**0.421**	0.615	**0.544**
	0.597		0.591		0.403		0.473	
2	0.484	**0.576**	0.430	**0.400**	0.410	**0.456**	0.542	**0.535**
	0.667		0.370		0.501		0.527	
3	0.551	**0.475**	0.492	**0.454**	0.513	**0.545**	0.713	**0.633**
	0.399		0.416		0.577		0.553	
4	0.357	**0.472**	0.626	**0.554**	0.428	**0.507**	0.648	**0.578**
	0.586		0.482		0.585		0.507	
5	0.536	**0.626**	0.622	**0.590**	0.553	**0.612**	0.568	**0.499**
	0.716		0.557		0.671		0.430	
6	0.709	**0.699**			0.511	**0.516**		
	0.689				0.520			
	Mean	**0.578**	**Mean**	**0.522**	**Mean**	**0.509**	**Mean**	**0.558**
	SD	0.091	**SD**	0.091	**SD**	0.067	**SD**	0.051
	SE	0.037	**SE**	0.041	**SE**	0.027	**SE**	0.023

PI3 Kinase western blot raw data

Band	Control	Chronic	Acute	A+C
1	0.096	0.134	0.150	0.123
2	0.103	0.106	0.083	0.084
3	0.104	0.066	0.081	0.099
4	0.064	0.061	0.073	0.089
5	0.094	0.082	0.073	0.106
6	0.057	0.076	0.068	0.115
7	0.046	0.084	0.068	0.127
8	0.066	0.075	0.070	0.108
9	0.100	0.078	0.068	0.112
10	0.122	0.110	0.053	0.089
11	0.115		0.077	
12	0.074		0.092	

Sample	Control		Chronic		Acute		Acute + Chronic	
	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean	Duplicates	Mean
1	0.096	**0.100**	0.134	**0.120**	0.150	**0.117**	0.123	**0.104**
	0.103		0.106		0.083		0.084	
2	0.104	**0.084**	0.066	**0.064**	0.081	**0.077**	0.099	**0.094**
	0.064		0.061		0.073		0.089	
3	0.094	**0.094**	0.082	**0.079**	0.073	**0.071**	0.106	**0.111**
	discard		0.076		0.068		0.115	
4	discard	**0.066**	0.084	**0.080**	0.068	**0.069**	0.127	**0.118**
	0.066		0.075		0.070		0.108	
5	0.100	**0.111**	0.078	**0.094**	0.068	**0.068**	0.112	**0.101**
	0.122		0.110		discard		0.089	
6	0.115	**0.095**			0.077	**0.085**		
	0.074				0.092			
	Mean	**0.092**	**Mean**	**0.087**	**Mean**	**0.081**	**Mean**	**0.105**
	SD	0.015	**SD**	0.021	**SD**	0.019	**SD**	0.009
	SE	0.006	**SE**	0.010	**SE**	0.008	**SE**	0.004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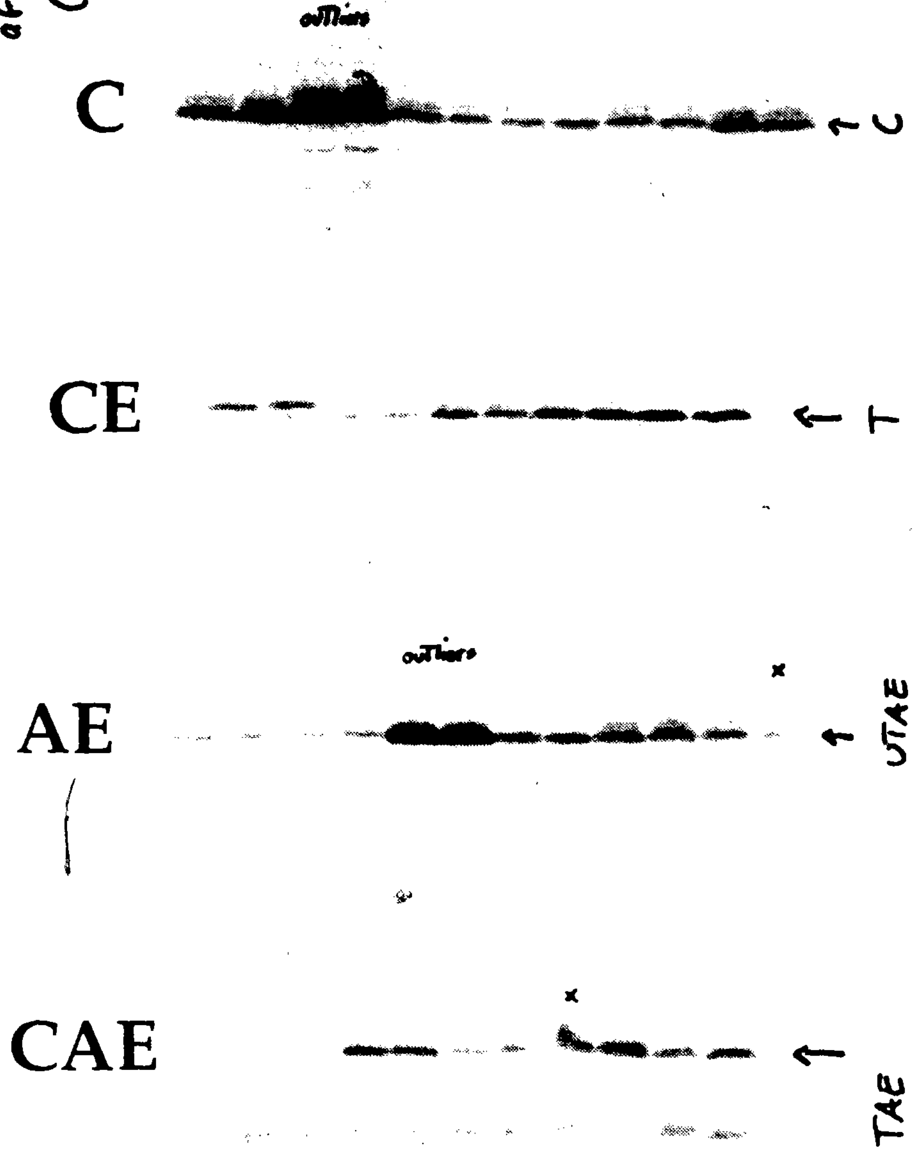
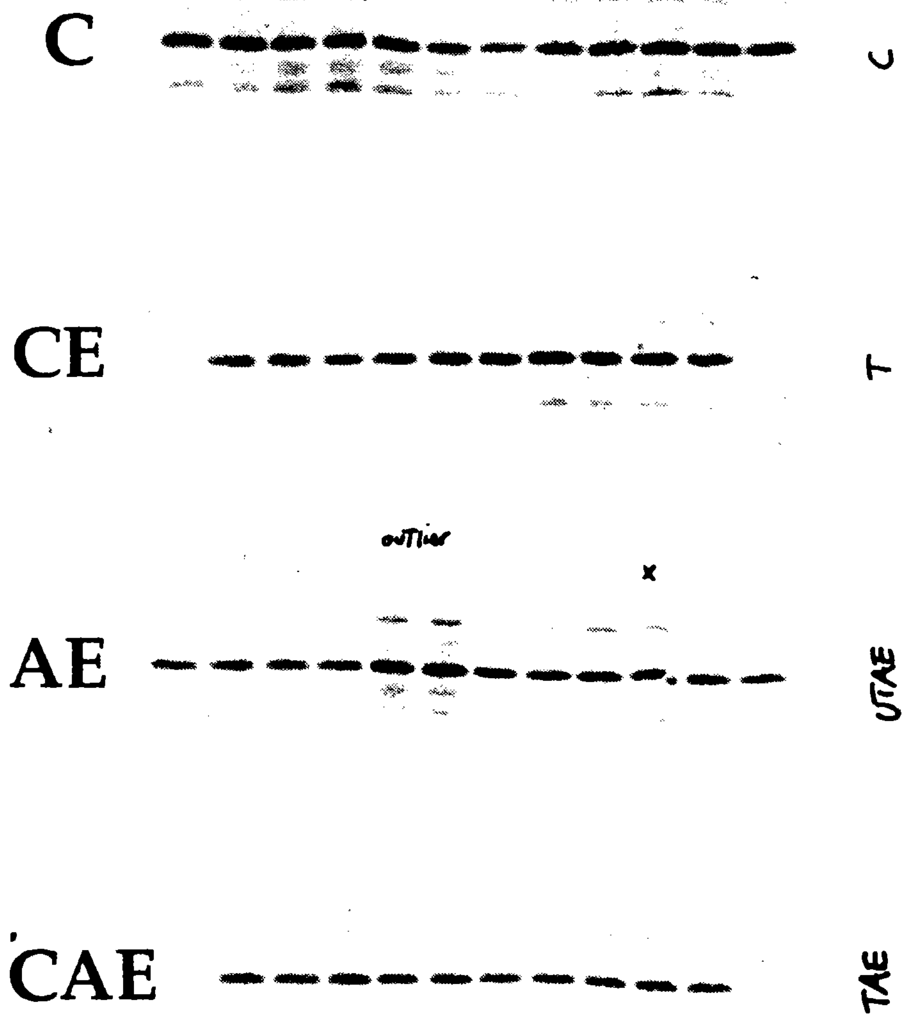
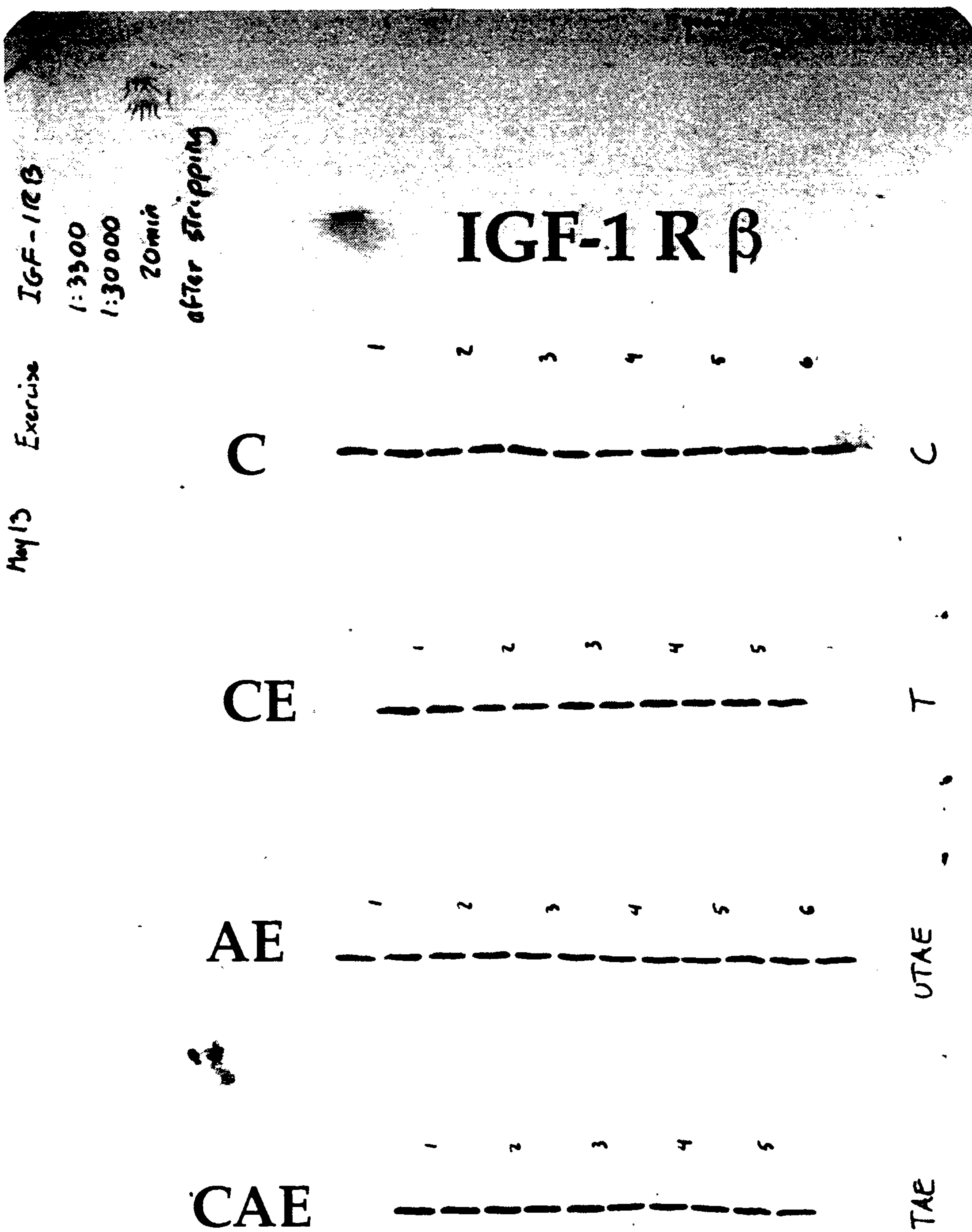
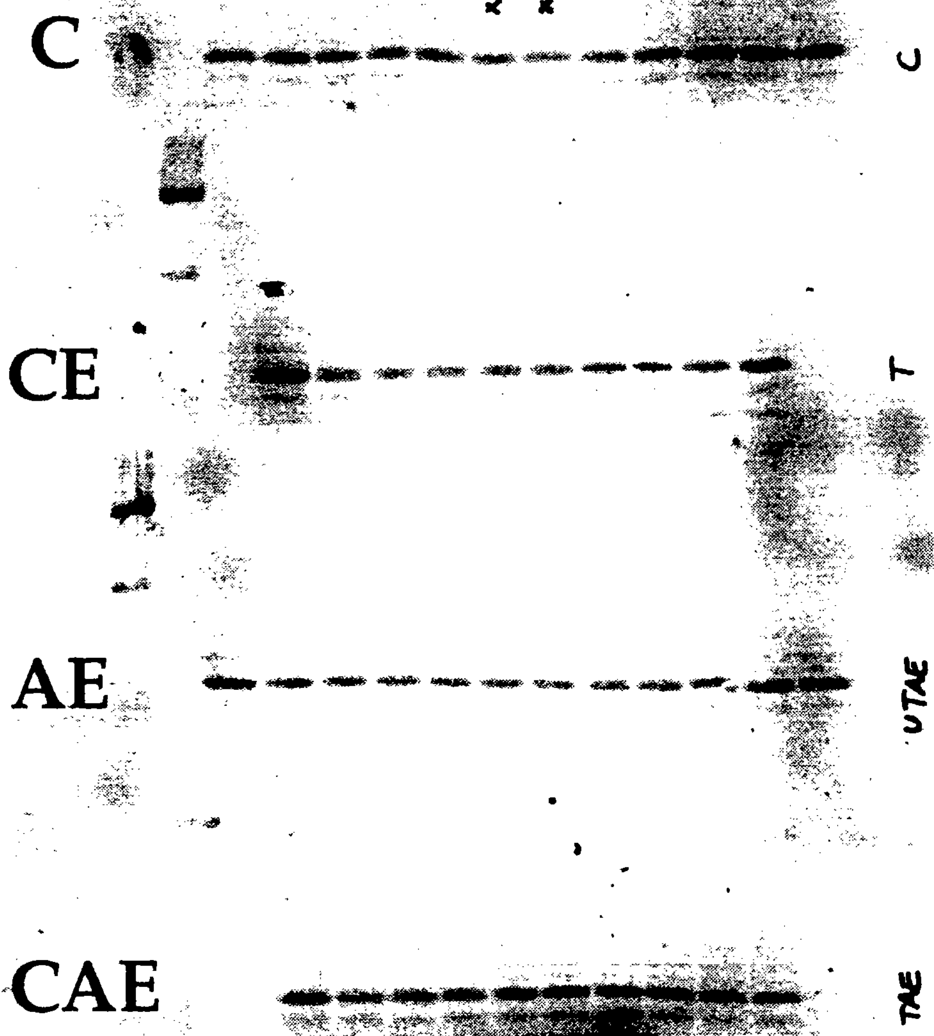
CE
AE
CAE
C
T
UTAE
TAE


Glut 4
C
CE
AE
CAE

Insulin R β




IGF-1 R β
C
CE
AE
CAE


p85 α
C
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C
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TAE

Appendix C

DETAILED METHODS

Contents:

- Western blot protocol
- Receptor autophosphorylation and substrate phosphorylation assay protocols
- Receptor radioligand binding assay protocol
- Immunoprecipitation protocol

WESTERN BLOT PROTOCOL

1. Tissue preparation

1.1 Isolation buffer
1.2 Tissue homogenization
1.3 Preparation of crude membrane extract
1.4 Bradford protein assay
  1.4.1 Procedure
  1.4.2 Bradford standard preparation

2. Electrophoresis

2.1 Sample preparation
  2.1.1 Sample buffer
2.2 SDS-PAGE stock solutions
2.3 Gel recipes
  2.3.1 5% stacking gel
  2.3.2 Running gel
2.4 Biorad Protean II usage and general notes
  2.4.1 Running gel preparation
  2.4.2 Stacking gel preparation
  2.4.3 Sample loading
  2.4.4 Electrophoresis run
  2.4.5 Running buffer
  2.4.6 Gel extraction
  2.4.7 Cutting gels
  2.4.8 Staining gels
  2.4.9 Drying gels

3. Electrotransfer

3.1 Membrane preparation
3.2 BioRad Transblot usage and general notes
  3.2.1 Transfer conditions
  3.2.2 Transfer buffer
3.3 After transfer

4. Immunodetection

4.1 Solutions
4.2 Immunodetection procedure
4.3 Antibody preparation
  4.3.1 Primary antibodies

4.3.2 Secondary antibodies
4.4 Membrane stripping

5. Densitometry

5.1 LKB UltroScan XL laser densitometer operation
5.1.1 Film positioning
5.1.2 Resolution
5.1.3 Scan width
5.1.4 Scan length
5.1.5 Smoothing
5.2 Pharmacia GelScan XL software operation and general notes
5.2.1 Data acquisition
5.2.2 Data analysis
5.2.3 Automating procedures
5.2.4 A note on background selection

1. Tissue Preparation

1.1 Isolation Buffer

100 mM N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES)
150 mM NaCl
5 mM ethylenediaminetetraacetic acid (EDTA)
5 mM $MgCl_2$
1% (wt/vol) Triton X-100
2% (wt/vol) Nonidet P-40 or Igepal CA-630
pH 7.6 (25°C)

protease inhibitors:

2 mM phenylmethylsulfonyl fluoride (PMSF)
60 μM leupeptin
60 μM pepstatin
1.5 mg/ml bacitracin (approx. 100 IU/ml)
3 mg/ml aprotinin (approx. 1 TIU/ml)
1 mg/ml benzamidine
1 mg/ml Nα-p-tosyl-l-arginine methyl ester (TAME)
1 mg/ml Nα-benzoyl-l-arginine ethyl ester (BAEE)

phosphatase inhibitors:

100 μM sodium orthovanadate Na_3VO_4
10 mM sodium fluoride NaF
10 mM sodium pyrophosphate $Na_4P_2O_7$

Notes:

1) Work from 10% stock solutions for Triton X-100 and NP-40
2) Prepare 50 ml of 2X concentrate without inhibitors
3) Aliquot inhibitors for a final volume of 10 ml 1X concentrate. On day of homogenization, prepare 10 ml of isolation buffer by adding aliquoted inhibitors to 5 ml of 2X concentrate buffer and completing with water to 10 ml (rinsing inhibitor tubes)
4) Use Sigma Ultra grade reagents where possible

For 50 ml 2X concentrate isolation buffer (no inhibitors):

2.383 g HEPES
0.876 g NaCl
0.146 g EDTA (long to dissolve)

0.048 g $MgCl_2$
pH 7.6 (25°C)
10 ml Triton X-100 10% stock
20 ml NP-40 10% stock
complete to 50 ml with dd water
store 4°C

Inhibitor Recipes (Aliquoted for final volume of 10ml):

PMSF (MW 174.2)	dissolve 34.84 mg in 5 ml 100% ethanol add 5ml dd water Aliquots of 1 ml, store 4°C, only keep 3 weeks ∴ 2 mM in 10 ml final
Leupeptin (MW 475.6)	10 mg in 10 ml dd water (directly into vial) Aliquots of 300 μl, store -20°C ∴ 60 μM in 10 ml final
Pepstatin (MW 685.9)	5 mg in 5 ml dd water (directly into vial) Aliquots of 400 μl, store -20°C ∴ 60 μM in 10 ml final
Bacitracin	Dilute to 10 000 IU/ml in dd water Aliquots of 100 μl, store -20°C ∴ 1000 IU in 10 ml final
Aprotinin	Dilute to 20 TIU/ml in dd water Aliquots of 500 μl, store -20°C ∴ 10 TIU in 10 ml final
Benzamidine	200 mg in 2 ml dd water Aliquots of 100 μl, store -20°C ∴ 10 mg in 10 ml final
TAME	200 mg in 2 ml dd water Aliquots of 100 μl, store -20°C ∴ 10 mg in 10 ml final
BAEE	200 mg in 2 ml dd water Aliquots of 100 μl, store -20°C ∴ 10 mg in 10 ml final

Na_3VO_4 (MW 183.9)	18.39 mg in 10 ml dd water Aliquots of 100 µl, store -20°C ∴ 100 µM in 10 ml final
NaF (MW 41.99)	41.9 mg in 1 ml dd water Aliquots of 100 µl, store -20°C ∴ 10 mM in 10 ml final
$Na_4P_2O_7$ (MW 446.1)	446 mg in 2 ml dd water Aliquots of 200 µl, store -20°C ∴ 10 mM in 10 ml final

1.2 Tissue Homogenization

1) Clean homogenizer probe in 0.1N NaOH and rinse in dd water
2) Keep samples, buffer, and homogenization vials on ice
3) Cool homogenizer probe in ice water
4) Measure and record tissue weight
5) Homogenize in 15 volumes (µl/mg) of ice-cold buffer, keeping approximately half of volume to rinse probe and vial
6) Homogenize 2x 10 seconds at 80% output using a Janke & Kunkel homogenizer with a 10 mm diameter probe. Minimize probe temperature increase.
7) Transfer homogenate to polystyrene test tube and cap
8) Clean, rinse and cool probe between samples.

1.3 Preparation of crude membrane extract

1) Centrifuge sample at 10 000 x g 20 min 4°C
2) Designate supernatant S1 and pellet P1
3) Freeze P1 at -80°C
4) Transfer S1 to polycarbonate ultracentrifuge tubes using fine tip transfer pipet
5) Incubate S1 20 min 4°C with frequent gentle vortexing
6) Centrifuge S1 150 000 x g 60 min 4°C
7) Designate supernatant S2 and pellet P2
8) Freeze P2 at -80°C
9) Transfer S2 to cryotube using fine tip transfer pipet
10) Measure and record exact volume of S2 with Hamilton syringe
11) Remove 10 µl aliquot of S2 for Bradford Protein Assay
12) Freeze S2 at -80°C

1.4 Bradford Protein Assay

1.4.1 Procedure

1) Use 5 ml polystyrene test tubes for assay
2) Dilute 10 µl aliquot of S2 in 2 ml dd water
3) Take 100 µl aliquot, add 700 µl dd water
4) Prepare BSA standards from 0 to 12 µg BSA in 800 µl
5) Add 200 µl of undiluted BioRad Protein Assay Reagent to sample and standard tubes using repeater pipetter
6) Cap tubes and invert gently, avoiding bubble formation (vortexing not recommended)
7) Allow exactly 10 min. for color development. Keep time consistent between assays.
8) Transfer tube contents (1 ml) to disposable optical cuvettes
9) Read at 595 nm using spectrophotometer
10) Backcorrect spectrophotometer using 0 µg standard tube
11) Use 2 series of standards for each assay
12) Plot optical density versus protein concentration for averaged standard data
13) Generate linear equation from standard curve and calculate protein concentration in original samples

1.4.2 Bradford Standard Preparation

1) Dilute 20 mg BSA in 400 ml dd water for a concentration of 1 µg/20 µl
2) Prepare in 5 ml polystyrene test tubes the following points:

0 µg:	0µl BSA	+ 800 µl dd water
2 µg:	40 µl	+ 760 µl
4 µg:	80 µl	+ 720 µl
6 µg:	120 µl	+ 680 µl
8 µg:	160 µl	+ 640 µl
10 µg:	200 µl	+ 600 µl
12 µg:	240 µl	+ 560 µl

3) Cap tubes and freeze at -20°C

2. Electrophoresis

2.1 Sample Preparation

- For all Western blots performed, samples are prepared to a protein concentration of 200 μg per 125 μl (1.6 mg/ml) using protein concentration data generated from Bradford assays
- Calculated volumes of S2 are diluted in an equal volume of 2x concentrate sample buffer and the final volume is completed by adding 1x sample buffer
- Diluted samples are boiled 4 min. in locking microcentrifuge tubes
- 200 μg of protein are loaded per lane, in duplicate.

2.1.1 Sample Buffer

60 mM trishydroxymethylaminomethane (Tris)
2% (wt/vol) sodium dodecyl sulphate (SDS)
10% (vol/vol) glycerol
5% (vol/vol) β-mercaptoethanol
pH 6.8 (25°C)

note:

- Prepare 2x concentrate
- Work from 10% SDS stock solution (stored RT°)
- Handle β-mercaptoethanol under fumehood

For 25 ml 2x concentrate Sample Buffer:

363 mg Trizma base (electrophoresis grade)
10 ml SDS 10% solution
5 ml glycerol
2.5 ml β-mercaptoethanol
pH 6.8 (25°C)
complete to 25 ml with dd water
store 4°C

2.2 SDS-PAGE Stock Solutions

A system of acrylamide and tris stock solutions facilitates preparation of gels of various acrylamide concentrations. Solutions for running gel are referred to as "lower" and solutions for stacking gel as "upper".

Note:

- Use electrophoresis grade reagents where possible
- Work from 10% SDS stock solution
- For improved safety, use BioRad premixed 40% acrylamide solution

Lower Tris Stock Solution

1.5 M Tris
0.4% SDS
pH 8.8 (25°C)

For 250 ml:

45.41 g Trizma Base
10 ml SDS 10% solution
pH 8.8 (25°C)
complete to 250 ml with dd water
store 4°C

Upper Tris Stock Solution

1 M Tris
0.4% (wt/vol) SDS
pH 6.8 (25°C)

For 100 ml:

12.11 g Trizma Base
4 ml SDS 10% solution
pH 6.8 (25°C)
complete to 100 ml with dd water
store 4°C

Lower Acryl Solution

32% (wt/vol) acrylamide
0.43% (wt/vol) bis-acrylamide

For 250 ml:

200 ml acrylamide 40% solution
1.075 g bis-acrylamide
allow bis to dissolve 15 minutes
complete to 250 ml with dd water
store 4°C

Upper Acryl Solution

20% (wt/vol) acrylamide
0.52% (wt/vol) bis-acrylamide

For 100 ml:

50 ml acrylamide 40% solution
0.52 g bis-acrylamide
allow bis to dissolve 15 minutes
complete to 50 ml with dd water
store 4°C

2.3 Gel Recipes

Prepare fresh 10% (wt/vol) stock solution of ammonium persulfate (APS) in microcentrifuge tube, approximately 100 mg in 1 ml

2.3.1 5% Stacking Gel (for 2 gels)

5 ml Upper Tris
5 ml Upper Acryl
10 ml water
80 µl ammonium persulfate 10% solution
50 µl Temed (N,N,N',N'- tetra-methylethylenediamine)

2.3.2 Running Gel (72 ml for 2 gels 11.5 cm long, 1.5 mm thick)

	8%	10%	12%	15%
Lower Tris	18 ml	18 ml	18 ml	18 ml
Lower Acryl	18 ml	22.5 ml	27 ml	34 ml
Water	36 ml	31.5 ml	27 ml	20 ml
10% APS	144 µl	144 µl	144 µl	144 µl
Temed	44 µl	44 µl	44 µl	44 µl

2.4 Biorad Protean II Usage and General Notes

2.4.1 Running Gel Preparation

1) Clean glass plates with ethanol and Kimwipes
2) Assemble glass sandwich using 1.5 mm spacers
3) Use a spare spacer to ensure proper spacer alignment
4) Inspect bottom of sandwich for perfect glass-spacer-glass interface
5) Place a reference mark at 11.5 cm from bottom of assembly
6) Make sure casting stand is level
7) Prepare running gel according to recipe; mix solution thoroughly before adding APS and Temed; do not degas
8) Tilt casting stand toward you to facilitate pouring of gel
9) Pour to reference mark
10) Use 10 cc syringe to very gently add 1 cm of water to top of gel in order to displace bubbles and prevent drying of gel
11) Let gel polymerize 1 hr at 25°C; keep remaining solution in beaker to confirm polymerization
12) After polymerization, pour out water layer and proceed to stacking gel preparation

2.4.2 Stacking Gel Preparation

- If preparing multiple sets of two gels, pour stacking gels separately for each set

1) Prepare stacking gel according to recipe; mix solution thoroughly; add APS and Temed only when ready to pour gel; do not degas
2) Insert clean 20 lane comb into center of glass sandwich
3) Use 10 cc syringe to carefully pour stacking gel around comb
4) Dislodge any bubbles caught under comb by gently moving comb
5) Pour gel right to top of glass
6) Let gel polymerize 10 min. at 25°C
7) Remove combs gently
8) If any lanes become misaligned during comb removal, use Hamilton syringe to reposition
9) Use 500µl Hamilton syringe to immediately drain remaining fluid from lanes

2.4.3 Sample Loading

- Use gel loading tips
- Withdraw tip as lane is being filled to minimize bubble formation
- If loading less than 125 µl, complete volume with sample buffer
- Fill empty lanes with sample buffer
- In an empty lane, load 25 µl of Amersham Rainbow molecular weight markers diluted in equal volume of 2x sample buffer; complete with sample buffer
- In an empty lane, load 20 µl of 0.1% bromophenol blue solution; complete with sample buffer

2.4.4 Electrophoresis Run

- Use deionized water only for running buffer (approx. 18 MΩ cm)
- Add approx. 2 L of running buffer to lower chamber of Protean II cell
- Carefully pour approx. 350 ml into upper chamber. Do not displace samples.
- All runs are performed overnight at RT° at a constant current of 22 mA per set of 2 gels
- Current is set to approximately 50 mA per set of 2 gels until dye front reaches running gel.
- Run is terminated when dye front reaches 1 cm from the bottom of glass sandwich

2.4.5 Running Buffer

25 mM tris
192 mM glycine
0.1% (wt/vol) SDS
pH 8.3 (25°C)

Note: BioRad premixed 10x concentrate T/G/SDS buffer is used for all runs.

2.4.6 Gel Extraction

1) Gently pry glass plates apart using the spacers
2) Pull large plate off leaving gel on small plate (logical orientation)
3) Avoid moving or distorting the gel
4) Cut off stacking gel using scalpel
5) Handle gel very carefully to avoid tearing

2.4.7 Cutting Gels

In order to simultaneously analyze large numbers of samples run on multiple gels, it is necessary to cut out the area of interest from each gels and transfer them simultaneously onto a single membrane. The use of colored molecular weight markers facilitates the process by providing reference points for cutting without requiring staining.

1) With the gel sitting on the small glass plate, pour a small layer of transfer buffer onto the surface to lubricate cutting
2) Press a clean plastic ruler onto gel surface at level of desired cut
3) Use a scalpel with a fresh blade
4) Do not make continuous cuts across gel, but rather multiple small cuts down into gel

2.4.8 Staining Gels

The following is a rapid protocol for non-quantitative staining of a gel. It is often used to confirm transfer efficency. Sensitivity is in the μg range.

Coomassie Blue R Staining solution:	0.1% Coomasie Blue R-250 dye 40% Ethanol 10% Acetic acid, glacial complete with dd water
50% Destaining solution:	50% Ethanol

	10% Acetic acid, glacial complete with dd water
20% Destaining solution	20% Ethanol 10% Acetic acid, glacial complete with dd water

note: use a closed container (such as Tupperware) for the following steps

1) Soak gel 30 min in staining solution. Staining solution may be reused.
2) Soak gel 2 hours in 50% destaining solution. Solution may be changed.
3) Soak gel 2 hours in 20% destaining solution. Solution may be changed.
4) Dry gel (see 2.4.9)

2.4.9 Drying Gels

Stained gels or colored molecular weight ladders can be dried and preserved using a BioRad Gel Dryer.

1) Place gel onto a wet piece of filter paper
2) Place filter paper onto drying apparatus and cover with a sheet of Saran Wrap cut to fit filter paper.
3) Cover everything with dryer's vacuum membrane
4) Turn on vacuum source (pump or faucet) and ensure good seal
5) Close lid
6) Set heat to spike and level out to 80°C
7) Set timer to 2 hrs

3. Electrotransfer

3.1 Membrane Preparation

- Use Millipore Immobilon-P 0.45 µm PVDF membrane
- Always handle membrane with gloves and tweezers if possible. Use flat bladed tweezers and handle by corners
- Cut membrane to maximum size of 15x20 cm; 12x16 cm is sufficient to transfer a complete gel
- Use a lead pencil to mark upper left corner of inside surface of roll
- Wet membrane in methanol for a few seconds until completely wet
- Rinse in water 5 minutes

3.2 BioRad TransBlot Usage and General Notes

1) Use large rectangular plastic basin for transfer sandwich assembly
2) Place one fibre pad followed by one piece of thick filter paper centered on each side of sandwich apparatus, and immerse in transfer buffer
3) Place PVDF membrane face up on center of filter paper on clear side of sandwich
4) Soak gel in transfer buffer 1 minute
5) Place gel onto center of membrane, upper left corner aligned with upper left corner of membrane
6) Place second filter paper followed by fibre pad onto gel
7) Close and lock sandwich and insert into transfer apparatus, gel (black side of sandwich) facing the negative electrode

3.2.1 Transfer Conditions

- Use deionized water only for transfer buffer (approx. 18 MΩ cm)
- All transfers performed for 3 hrs at 4°C at approximately 60 V (450 mA)
- Transfer buffer is pre-cooled to 4°C
- Stir bar and magnetic stir plate is used throughout transfer
- Normal transfer buffer contains 10% methanol, added immediately prior to transfer
- Less methanol may be used and 0.02% SDS may be added for enhanced transfer of high molecular weight proteins.

3.2.2 Transfer Buffer

25 mM tris
192 mM glycine
10% (vol/vol) methanol
pH 8.3 (25°C)
Note: BioRad premixed 10x concentrate T/G buffer is used for all transfers.

For 4 L of transfer buffer:

400 ml 10x concentrate buffer
400 ml methanol
3200 ml dd water

3.3 After Transfer

1) Rinse membrane in water 5 minutes
2) Soak in Ponceau S solution (Sigma) 5 minutes or until protein bands are visible

3) Remove from dye solution and rinse excess twice with small volumes of water
4) Place membrane on glass plate, cover with Saran Wrap, and photocopy
5) Rinse membrane in water 5 minutes
6) Rinse in TBST solution 5 minutes
7) Let membrane air dry on filter paper or proceed to immunodetection

4. Immunodetection

4.1 Solutions

Tris-Buffered Saline + Triton (TBST)	**For 3 L of 10x concentrate**
50 mM tris	227.4 g Trizma Pre-Set 7.4
150 mM NaCl	263 g NaCl
0.5% (vol/vol) Triton X-100	150 ml Triton X-100
pH 7.4 (25°C)	pH n/a
	complete with dd water to 3 l
	store RT°

Tris-Buffered Saline (TBS)	**For 3 L 10x concentrate**
50 mM tris	227.4 g Trizma Pre-Set 7.4
150 mM NaCl	263 g NaCl
pH 7.4 (25°C)	pH n/a
	complete with dd water to 3 L
	store RT°

- Use 1000 ml graduated cylinder with stir bar to dilute concentrate

4.2 Immunodetection Procedure

1) If working from a dry blot, membrane must be activated by immersing in methanol for 5 sec. or until fully wet, then rinsed 5 min. in water
2) Wash 5 min. at RT° in TBST
3) Block non-specific binding sites of membrane 90 min. at RT° with a 3% BSA (fraction V) solution in TBST under gentle agitation. Use 50 ml in a heat-sealed plastic bag on an orbital shaker.
4) Wash 3x 15 min. at RT° in TBST with moderate agitation
5) Incubate overnight at 4°C in primary antibody solution (1% BSA, 0.5% NaN_3, TBST) with gentle agitation. Use a heat-sealed plastic bag on an orbital shaker.
6) Wash 5x 15 min. at RT° in TBST with moderate agitation
7) Incubate 90 min. at RT° in secondary antibody solution (1% BSA, TBST) with gentle agitation. Use a heat-sealed plastic bag on an orbital shaker.
8) Wash 3x 10 min. in TBST with moderate agitation
9) Wash 3x 10 min. in TBS with moderate agitation. All detergent must be removed before proceeding.
10) Drain and submerge membrane in fresh Amersham ECL mixture 1 min.
11) Drain and place membrane on glass plate. Cover with Saran Wrap.
12) Place plate in cassette and expose to Amersham ECL Hyperfilm.
13) Remove film, fold upper right corner
14) Immerse with exposed surface up in Kodak D-19 developer solution for approx. 1 min.
15) Rinse film by very briefly immersing in water and immerse in fixer/hardener approx. 5 min.
16) Rinse film in water at least 15 min. and hang to dry
17) Wash membrane 5 min. in TBST and air dry on filter paper

additional notes:

- Procedure may be halted at any TBST step and blot dried or stored at 4°C in TBST
- For full-sized blots (15x20 cm), use 50 ml of antibody solution, 100-150 ml of wash solution and 30 ml of ECL mixture (15 ml + 15 ml)
- Use plastic sheets and food bag heat-sealer to make plastic bags

4.3 Antibody Preparation

4.3.1 Primary Antibodies

Primary Ab	Type	Manufacturer	Concentration
anti-insulin receptor β subunit (29B4)	mouse monoclonal IgG_1	Santa Cruz Biotech SC-009	1: 10000
anti-IGF-1 receptor β subunit (C-20)	rabbit polyclonal IgG	Santa Cruz Biotech SC-713	1: 5000
anti-GLUT-4 (E-20)	goat polyclonal IgG	Santa Cruz Biotech SC-1607	1: 5000
anti-GLUT-4 (C-20)	goat polyclonal IgG	Santa Cruz Biotech SC-1608	1: 5000
anti-GLUT-4	rabbit polyclonal	East Acres Biologicals	1: 3300
anti-GLUT-1	rabbit polyclonal	East Acres Biologicals	1: 3300
anti-phosphotyrosine (PY69)	mouse monoclonal IgG_{2a}	Santa Cruz Biotech SC-021	1: 1000
IRS-1 (C-20)	rabbit polyclonal IgG	Santa Cruz Biotech SC-559	1:5000
anti-p85 α subunit	rabbit polyclonal	Santa Cruz Biotech	1:5000

- Primary antibodies are normally prepared in 1:100 stocks in TBST, 1% BSA, and 0.5% NaN_3.
- Work from 10% BSA in TBST solution (aliquoted to 10 ml and stored -20°C) and 5% NaN_3 (in water) solution (100 ml stored at 4°C)

For 3 ml stock:

300 µl TBST 10x concentrate
300 µl Fraction V BSA 10% solution in TBST
300 µl NaN_3 5% solution
add 30 µl antibody

complete to 3 ml with dd water
store 4°C

Working solutions are prepared from 1:100 stocks in TBST, 1% BSA, and 0.5% NaN_3

For 50 ml solution:

5 ml TBST 10x concentrate
5 ml Fraction V BSA 10% solution in TBST
5 ml NaN_3 5% solution
appropriate volume of antibody stock
complete to 50 ml with dd water
store 4°C

Primary antibody stocks and working solutions may be kept at 4°C for weeks and reused several times.

4.3.2 Secondary Antibodies

Secondary Ab	Type	Manufacturer	Concentration
anti-goat HRP conjugated	donkey polyclonal IgG	Santa Cruz Biotech SC-2020	1: 30000
anti-rabbit HRP conjugated	goat polyclonal IgG	Santa Cruz Biotech SC-2004	1: 30000
anti-mouse HRP conjugated	goat polyclonal IgG	Santa Cruz Biotech SC-2005	1: 30000

- Secondary antibodies are normally prepared in 1:300 stocks in TBST and 1% BSA without sodium azide
- Work from 10% BSA in TBST solution (aliquoted to 10 ml and stored -20°C)

For 3 ml stock:

300 µl TBST 10x concentrate
300 µl Fraction V BSA 10% solution in TBST
add 10 µl antibody
complete to 3 ml with dd water
store 4°C

Working solutions are prepared from 1:300 stocks in TBST and 1% BSA without sodium azide.

For 50 ml solution:

5 ml TBST 10x concentrate
5 ml Fraction V BSA 10% solution in TBST
0.5 ml of antibody stock
complete to 50 ml with dd water
Secondary antibody stocks can be kept at 4°C for days. Secondary antibody solutions may be used only once as incubation is normally performed at room temperature. Sodium azide is not compatible with HRP catalyzed reactions.

4.4 Membrane Stripping

- Necessary for removal of antibodies
- Incubate blot 30 min. 50-70°C with occasional agitation
- Block membrane and repeat immunodetection procedure

Stripping Buffer	**For 1 L:**
62.5 mM tris	9.85 g Trizma-HCl
100 mM β-mercaptoethanol	7 ml β-mercaptoethanol
2% (wt/vol) SDS	200 ml SDS 10% solution
pH 6.7 (25°C)	PH 6.7 (25°C)
	complete to 1 L with dd water
	store 4°C

5. Densitometry

All quantification of films was performed by laser densitometry using an LKB UltroScan XL densitometer and Pharmacia GelScan XL software running on a 386DX PC compatible computer.

5.1 LKB UltroScan XL laser densitometer operation

The densitometer's configuration is adjusted through the instrument's computer interface but operation of the densitometer is conducted remotely through GelScan software. All raw data files are saved to computer hard drive. Data is expressed as 0-4 Absorbance Units in 0.01 AU increments.

5.1.1 Film positioning: Films are oriented so as to scan bands perpendicular to their long axis and to automate scanning of an entire row. Up to 16 bands are programmed for automated scanning, each band generating a separate data file. The X position is aligned with the center of the band.

5.1.2 Resolution: Resolution is set to 20μm steps for all scans. (Y-step=0)

5.1.3 Scan width: Each band is scanned twice on either side of the center line with an 800μm wide line beam and no overlap, giving a total scan width of 3.2 mm. The mean of the four passes is saved to disk. (X-width=4)

5.1.4 Scan length: Length of scan is set to 8mm in order to scan some background area above and below bands. (Y-axis=8mm)

5.1.5 Smoothing: No smoothing is used for films with high signal to noise ratio. For films of poorer quality, smoothing is set to 1 or 2.

Note: It is important to clean lower glass plate and diffuser screen regularly. These optical elements should be replaced when scratched.

5.2 Pharmacia GelScan XL software operation and general notes

GelScan XL is used to remotely control the densitometer, acquire data and analyze data files. Since each band on a film corresponds to a separate data file, a data file normally contains only one peak of interest. Using the Evaluate function, a background as well as a peak start and peak end point are defined for each data file. Integration of the area under the curve of the peak minus the background is performed. The result of the integration and the analyzed curve are output to a laser printer. For films with high signal to noise ratio, the analysis and output are automated using the Autorun function.

5.2.1 Data acquisition:

1) Login to GelScan
2) Place film in densitometer.
3) Configure and program densitometer as detailed in section 5.1
4) Enter "Scan" function in GelScan
5) Name file to be acquired. For automated acquisition, files will be given same name with sequential numbers. Data files are given a "d" extension.
6) Confirm entries and begin scanning.

7) After scanning, remove files from default directory and place in user directory using dos shell or windows.

5.2.2 Data analysis:

1) Login to GelScan
2) Enter "Evaluate" function
3) Activate the "File" menu and select the appropriate directory and data file using "Load".
4) Select a method file if available.
5) Hit return to display the data file graphically.
6) If no method file was selected, activate the "Parameter" menu to select background and peak search options.
7) Set peak Number to "1", Background to "horizontal", and Integration to "signal". Use default values for other parameters.
8) Exit "Parameter" and activate the "Integration" menu to integrate area under curve. Integrated optical density for a peak is expressed as AU x mm.
9) Use the "Adjust" menu to override automatic peak selection and peak start and end points, if necessary.
10) "Print" curve and results table to laser printer.
11) Load next data file.

5.2.3 Automating procedures:

1) In order to avoid configuring the parameters for each analysis, a method file containing the appropriate parameters can be created using the "Method" menu.

2) For films with high signal to noise ratio, the entire analysis can be automated using the "Autorun" menu. "Autorun" requires a methods file and works best with "Background" set to "valley". It is essential to examine all outputed curves for proper peak start and end point selection and background placement. Any improperly analyzed curve must be reanalyzed manually.

5.2.4 A note on background selection:

Background can be determined manually or automatically using one of three algorithms. It is recommended to use automatic determination in order to keep the analysis process consistent.

If the film background is very consistent and the scanned area in a data file includes both signal and background, the "horizontal" option is

adequate as it rejects the lowest 5% of the data values. When the background is different above the band of interest as compared to below, then the "valley" option is recommended as it draws a line between the peak start and end points and rejects all data below the line. On high signal to noise ratio films, the "valley" option produces results nearly identical to horizontal. "Valley" is the preferred selection when using "Autorun".

Receptor autophosphorylation and substrate phosphorylation assay protocols

The following is the detailed procedure for a non-radioactive western blot-based maximal phosphorylation assay of insulin or IGF-1 receptor autophosphorylation and substrate phosphorylation.

1. Sample Preparation
   1.1 Isolation Buffer

2. Procedure for receptor autophosphorylation assay

3. Procedure for receptor substrate phosphorylation assay

4. Ligand Preparation
   4.1 Insulin dilutions
   4.2 IGF-1 dilutions

5. Assay buffer

6. ATP/$MgCl_2$ solution

7. Substrate preparation

8. Western blot procedure special notes

1. Sample Preparation

A crude solubilized receptor preparation is used for the assay. 200µg of membrane protein is required per experiment.

Refer to Part 1 of Western blot protocol for tissue preparation.

1.2 Tissue homogenization
1.3 Preparation of crude membrane extract
1.4 Bradford protein assay

1.1 Isolation Buffer (same composition as for Western blot)

100 mM HEPES
150 mM NaCl
5 mM EDTA
5 mM $MgCl_2$
1% (wt/vol) Triton X-100
2% (wt/vol) Nonidet P-40 or Igepal CA-630
pH 7.6 (25°C)

protease inhibitors:
2 mM PMSF
60 µM leupeptin
60 µM pepstatin
1.5 mg/ml bacitracin (approx. 100 IU/ml)
3 mg/ml aprotinin (approx. 1 TIU/ml)
1 mg/ml benzamidine
1 mg/ml TAME
1 mg/ml BAEE

phosphatase inhibitors:
100 µM sodium orthovanadate Na_3VO_4
10 mM sodium fluoride NaF
10 mM sodium pyrophosphate $Na_4P_2O_7$

2. Procedure for receptor autophosphorylation assay

1) Incubate samples with ligand and buffer 12-18 hrs at 4°C with constant agitation.
2) After incubation, add ATP/$MgCl_2$ to start phosphorylation reaction.
3) Run reaction 60 min. at 4°C with constant agitation.
4) Stop reaction by boiling samples in sample buffer 5 min.
5) Run samples on SDS-PAGE gel. (Must run entire 200µg)
6) Refer to Western blot protocol for electrophoresis, electrotransfer, and immunoblotting.

Overnight pre-incubation:

20µl of 5x concentrate assay buffer
20µl of properly diluted ligand
60µl of sample + water (200µg protein)
vortex and place on orbital shaker at 4°C

Start reaction:

vortex
10µl of 10x ATP/$MgCl_2$ solution
vortex and place on orbital shaker at 4°C

Stop reaction:

50µl of 3x concentrate sample buffer
vortex
Boil 5 min
Load 150µl on gel

- Use locking microcentrifuge tubes for assay.
- Place tubes in covered tube holder and tape to an orbital shaker in cold room for overnight incubation and reaction.
- When working at bench, place tubes in tube holder and immerse in large ice bucket
- 150µl is appropriate volume for 20 lane gel

3. Procedure for receptor substrate phosphorylation assay

1) Incubate samples with ligand and buffer 12-18 hrs at 4°C with constant agitation.
2) After incubation, add substrate and ATP/$MgCl_2$ solution to start phosphorylation reaction.
3) Run reaction 60 min. at 4°C with constant agitation
4) Stop reaction by boiling samples in sample buffer 5 min.
5) Run samples on SDS-PAGE gel
6) Refer to Western blot protocol for electrophoresis, electrotransfer, and immunoblotting.

Overnight pre-incubation:

20µl of 5x concentrate assay buffer
20µl of properly diluted ligand
60µl of sample + water (200µg protein)
vortex and place on orbital shaker at 4°C

Start reaction:

vortex
10µl of 10x ATP/$MgCl_2$ solution
10µl of properly diluted substrate
vortex and place on orbital shaker at 4°C

Stop reaction:

60µl of 3x concentrate sample buffer
vortex
Boil 5 min
Load 36µl on gel (corresponding to 10µg histone 2b) and complete with 1x sample buffer

Note: Remainder of sample may be frozen and run later as a receptor autophosphorylation assay, albeit with 80% of recommended protein content. Initial amount of protein may be adjusted if both assays are to be run from same experiment.

4. Ligand Preparation

Prepare by serial dilution 6 concentrations of insulin or IGF-1 ranging from $1x10^{-11}$ M to $1x10^{-6}$ M.

- Concentrations are fixed for a final volume of 100μl and prepared in 20μl aliquots.
- Store all stock solutions and aliquots at -80°C

Concentration (M)	Insulin (ng in 100μl final)	IGF-1 (ng in 100μl final)
$1x10^{-6}$	600	750
$1x10^{-7}$	60	75
$1x10^{-8}$	6	7.5
$1x10^{-9}$	0.6	0.75
$1x10^{-10}$	0.06	0.075
$1x10^{-11}$	0.006	0.0075

4.1 Insulin dilutions

1) Work from a 10mg in 10ml stock
2) From the stock, take 30μl and add 970μl water, resulting in 600ng in 20μl
3) From 2) take 100μl and add 900μl, resulting in 60ng in 20μl
4) From 3) take 100μl and add 900μl, resulting in 6ng in 20μl
5) From 4) take 100μl and add 900μl, resulting in 0.6ng in 20μl
6) From 5) take 100μl and add 900μl, resulting in 0.06ng in 20μl
7) From 6) take 100μl and add 900μl, resulting in 0.006ng in 20μl

note: Stock solution requires a small amount of acid in order to dissolve insulin

4.2 IGF-1 dilutions

1) Dilute 75μg in 1000μl
2) Take 200μl from stock and freeze remainder
3) To 200μl add 200μl water, resulting in 750ng in 20μl
4) From 3) take 40μl and add 360μl, resulting in 75ng in 20μl
5) From 4) take 40μl and add 360μl, resulting in 7.5ng in 20μl
6) From 5) take 40μl and add 360μl, resulting in 0.75ng in 20μl

7) From 6) take 40µl and add 360µl, resulting in 0.075ng in 20µl
8) From 7) take 40µl and add 360µl, resulting in 0.0075ng in 20µl

5. Assay buffer

50 mM HEPES
5 mM $MnCl_2$
100 µM sodium orthovanadate
0.1 % BSA
0.1 % Triton X-100
pH 7.4 (25°C)
store 4°C

- Prepare 50 ml assay buffer in 5x concentrate

6. ATP/$MgCl_2$ solution

1mM ATP
10 mM $MgCl_2$
pH 7.4 (25°C)
store -20°C

- Prepare ATP/$MgCl_2$ solution in 10x concentrate and aliquot in 1.5 ml microcentrifuge tubes to avoid repeated freeze-thaw

7. Substrate preparation

Histone 2b is used as a substrate for insulin receptor and IGF-1 receptor tyrosine kinase activity. The molecular weight of histone 2b is slightly inferior to 20kDa, high enough to be separated on a 6% SDS-PAGE gel.

- Substrate must be used at a concentration of at least 0.5mg/ml.
- 1mg of lyophilisized Histone 2b (supplied by Boehringer Mannheim) is reconstituted in 200µl water and is sufficient for 20 experiments.

8. Western blot procedure special notes

- 8% running gels are used for the autophosphorylation assay
- 6% gels are used for the substrate phosphorylation assay
- When using ligand concentration points and a 0 point, two sample are run per gel. It is recommended to run samples from different groups on

each gel. All gels are simultaneously run and transferred to the same membrane to allow quantitative comparisons between samples run on different gels.

- Blots are probed with PY69 anti-phosphotyrosine antibody

Receptor radioligand binding assay procedure

1. Sample Preparation

A crude detergent-solubilized receptor preparation is used for the assay. 300μg of membrane protein is required per experiment.

Refer to Part 1 of Western blot protocol for tissue preparation.

1.1 Isolation buffer
1.2 Tissue homogenization
1.3 Preparation of crude membrane extract
1.4 Bradford protein assay

2. Buffers and solutions

2.1 Binding buffer:

25 mM HEPES
150 mM NaCl
1.5 mM $MgSO_4$
0.5 % BSA
100 U/ml bacitracin
75 mM N-acetylglucosamide
pH 8.0 (25°C)

2.2 Other solutions:

0.3% bovine γ-globulin in 0.04% Triton X-100
store at 4°C for 1-2 weeks

25% polyethylene glycol (PEG)

12.5% PEG

note: PEG dissolves only at >60°C

3. Ligand preparation

Prepare by serial dilution 7 concentrations of cold insulin or IGF-1 ranging from 1×10^{-12} to 1×10^{-6}. Concentrations are fixed for a final volume of 200μl and prepared in 60μl aliquots. Stocks and aliquots are stored at -80°C.

^{125}I-insulin or ^{125}I-IGF-1 is purchased as 2000 Ci/mmol powder. 5µCi is resuspended in 2500µl of 1 mM HCl. 100µl is added to 400µl binding buffer for a working concentration of approximately 1.4×10^{-11} M.

4. Procedure

1) Prepare 7 unlabelled ligand concentration points as well as a 0 point
2) Work with a final volume of 200µl:

   15µl ^{125}I-ligand
   60µl cold ligand
   25 µl diluted sample
   100µl binding buffer

3) Incubate overnight at 4°C with gentle agitation

After overnight incubation:

4) Add 100µl 0.3 % γ-globulin to precipitate receptor-ligand complexes (γ-globulin must be vortexed constantly)
5) Add 300µl 25% polyethylene glycol (PEG) solution
6) Vortex
7) Incubate 15 min at 4oC
8) Spin 10 min 10 000x g at 4oC
9) Discard supernatant into hot liquid waste
10) Add 300µl 12.5% PEG solution
11) Vortex
12) Spin 10 000x g at 4oC
13) Discard supernatant into hot liquid waste
14) Repeat 12.5% PEG wash
15) Evaporate remaining liquid under fume hood (in order to prevent contamination of counter tubes).
16) Place microcentrifuge tubes into γ counter tubes (If microcentrifuge tubes are too large, it may be necessary to slice off upper half with a hot knife)
17) Count activity in pellet using γ counter

Note: 1) Use locking microcentrifuge tubes.
2) Pipet small volumes onto a dry surface of microcentrifuge tube
3) Use gel tips for to reduce contamination error.
4) Do not contact pellet during removal of supernatant. Recentrifuge if any contact occurs. Leave a small amount of supernatant as a safety margin.

5. Analysis

1) Counts at 10-6 M is considered non-specific.
   Calculate specific CPM where specific CPM = Counts - nonspecific
2) The 0 M point is considered 100% bound
   Calculate % bound
   where % bound = (specific CPM/specific CPM at 0 M) x 100
3) Plot % bound over log scale of molarity
4) Calculate % free where % free = (100- (specific bound/total)) x 100
5) Calculate bound/free ratio
6) Calculate B/F over bound
   where bound = % bound x [cold] x final volume / [protein]

Immunoprecipitation procedure

1. Buffer A

0.1 M Tris
0.15 M NaCl
0.5 M $MgCl_2$
0.5% w/vol NP-40
pH 8.5 (25°C)

2. Protein A sepharose

- Protein A insolubilized on sepharose CL-4B (Sigma P3391)
- Binding capacity 20mg human IgG/ml
- 1g makes 4ml of slurry
- Normally require between 10% and 50% of the amount of sample.

3. Bead preparation

1) To 100mg Protein A sepharose in a microcentrifuge tube, add 1ml of buffer A
2) Gently inverse to wet Protein A. Do not vortex as Sephadex may detach from Protein A. Alternatively, use a pipet tip to stir gently.
3) Wait 15 min at room temp. for bead formation (sleury).
4) Spin 15 min at low speed (~1000x g)
5) Discard supernatant and keep slurry.
6) Add 1ml of buffer A and tap tube to resuspend beads.
7) Spin again 15 min.
8) Discard supernatant and keep slurry.
   (washes are needed to remove stabilizers - dextran and lactose)

4. Immunoprecipitation

Day 1:

1) Dilute sample with buffer A. (Estimate dilution so as to have desired amount of protein in 100µl of supernatant - see below)
2) Add ~25µl slurry (difficult to pipet)
3) Shake spin 2 minutes
4) Collect supernatant (this step cleans out non-specific material on Protein A)
5) Take 100µl of supernatant and add antibody at 1:10 concentration
6) Incubate overnight at 4°C on orbital shaker

Day 2:

7) Add 50µl Protein A sepharose slurry
8) Incubate overnight at 4°C on orbital shaker
   (Protein A will bind the antibody bound to the protein of interest)

Day 3:

9) Spin at low speed 5 min. 4°C
10) Discard supernatant
11) Add 500µl buffer A to tube and spin again.
12) Repeat wash 4 times
13) Resuspend final pellet in HEPES buffer *
14) Take aliquot for Bradford assay *
15) Boil in sample buffer * (estimate volume of pellet and add equal amount of 2x concentrate and complete with 1x)
16) Vortex briefly to break complex
17) Keep supernatant and discard sepharose
18) Run supernatant on SDS-PAGE gel *

Day 4:

18) Transfer to membrane and perform immunodetection with another antibody. *

* Refer to Western blot protocol

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3) The receptor autophosphorylation and substrate phosphorylation assay protocols presented here were developed by Louis Martineau.