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EXERCISE EFFECTS ON MUSCLE GLUCOSE UPTAKE AND INSULIN ACTION

A search for intracellular signalling pathways

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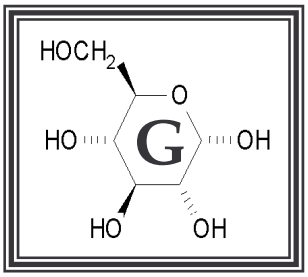
PREFACE



lucose was one of the pioneer molecules, responsible for the beginning of life on this beautiful planet. When the very first cell was born 3 billion years ago, there was no oxygen in the atmosphere. The 'Adam cell' derived the energy for survival anaerobically, presumably from breakdown of glucose (glycolysis). He was called 'cell' because he had made an outer membrane of fat (phospholipids), separating the interior of the cell from the environment. Glucose is like any other sugar a water-soluble compound (fat-insoluble), making it impossible to diffuse through a fat phase. Here, Adam cell was confronted with a fundamental problem : 'If he couldn't find a way to transport glucose into his interior through the fat membrane, he would die from starvation'. Adam cell survived and gave birth to the immense variety of cells, plants and animals we know today. In all animals, the unique ability to move around is made possible by the contractile properties of muscle cells. The increased need for energy during contractions prompted the first muscle cells with a new fundamental problem: 'How can they acutely regulate the entrance of glucose through the fat membrane, depending on the intracellular need for fuel?'

The transport of glucose through a cell membrane and its acute upregulation in working muscle cells are two intriguing mysteries in the understanding of our biologic heritage. They also form the basic questions in this PhD-thesis. A good way to approach this problem is to put yourself in the Creator's place and ask yourself: 'How would I have created a mechanism, allowing regulated transfer of glucose through a lipid membrane?' One could for example build small water-containing channels, through which the sugar molecules can 'swim' to the inside. It would also be possible to open and close the channels through regulation by a sensor, a gauge for the intracellular glucose need or abundance. However, a problem with such channels is that it would allow exchange with all water-soluble molecules, i.e. the cell membrane would leak everywhere and loose its function. Therefore, one should create channels, specific for the transfer of glucose. Adjusting the diameter to the size of the glucose molecules would solve the problem of the bigger ones, but not of the smaller. We already know that nature has almost perfectly solved this problem with a three-dimensional protein, forming a highly specific glucose transport channel, thereby allowing the transfer of D-glucose, but not L-glucose (the identical mirror image of D-glucose) or most other molecules. The problem of the need for opening and closing of the channel is overcome by simply removing the channel from the membrane, when it has to be inactivated (decreased need for

glucose). The mechanism of mobilisation of glucose transporters will be discussed in detail in the introductory chapters. The search for a sensor of intracellular energy needs and for the signal leading to mobilisation of glucose transporters has been the main focus of the experiments, described in the papers.

From the above, it is clear that nature is a master in problem solving. The extreme complexity of some of the intracellular signalling pathways that have evolved, is in sharp contrast with the simplicity of the tools that have led to them, the evolution by natural selection. The unravelling of the mysteries of biology is the biggest puzzle ever and it is extremely entertaining to work on it. Although our own contribution is very, very small, the mass effect of a joint effort of a million researchers all over the world, makes it go excitingly fast at the moment.

Wim Derave, October 1999

Put up in a place where it's easy to see

The cryptic admonishment T.T.T.

When you feel how depressingly slowly you climb,

it's well to remember that Things Take Time.

We glibly talk of nature's laws

but do things have a natural cause?

Black earth turned into yellow crocus

in undiluted hocus-pocus.

(‘Grooks’ by Piet Hein, a Danish poet and scientist)

CHAPTER 1: INTRODUCTION TO MUSCLE CARBOHYDRATE METABOLISM

'Il n'y a pas de question plus importante, en Physiologie générale, que l'étude des rapports existants entre le travail chimique et le travail mécanique des muscles'
(M.A. Chauveau, 1887)

Historical overview

1850. It was not until the middle of the 19th century that the fate of sugars within the body was examined. Early experiments by Schmidt (1844) and Claude Bernard (1848-1857) clarified that glucose is the main sugar present in the blood and that it can be utilised by the body [256][1]. Claude Bernard, the father of experimental physiology, identified in dogs that the liver can store glucose under the form of glycogen and secrete glucose in the blood [23;256]. Later, glycogen was also identified in muscle as an energy source for exercising muscles. However until 1865 it was still generally believed that the energy needed for muscular contraction originates from the breakdown of a portion of the muscle's own substance, protein. In 1840, Justus Liebig had proposed that the total amount of work performed by the body was proportional to the amount of nitrogen (originating from protein breakdown) excreted in the urine [44]. In 1866, after the completion of a mountain ascent without protein ingestion nor considerable high urinary nitrogen excretion this idea was challenged by Fick, Wislicenus and Frankland [44]. They concluded that protein cannot be the sole source of energy for muscular work and that non-nitrogenous material (i.e. carbohydrates and fat) are the best materials for the production of work [44].

1889. Chauveau makes one of the earliest and most important observations in exercise metabolism by collecting blood from exercising masticating muscles in four old horses, while eating hay [50]. He demonstrated that both blood flow is increased and more glucose is extracted from the blood in exercising muscles compared to resting muscles [50]. The search for the mechanism of exercise-stimulated glucose uptake by muscles has started.

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[1] Numbers between square brackets refer to an alfabetically ordered reference list at the end of this thesis

1921. In Toronto, Banting and Best succeeded to extract a hormone, called insulin, from the pancreas [16]. The hormone's main effect is a decrease in blood glucose levels. Shortly after its discovery, insulin is shown to stimulate glucose uptake by muscle and fat tissue and to inhibit liver glucose output [42;61]. The discovery of insulin is a major breakthrough in the understanding of metabolic regulation and in the treatment of diabetes [96].

1920-1940. The interbellum is one of the most fruitful periods in the study of metabolism during exercise [242]. Research took mainly place at the laboratory for the Theory of Gymnastics in Copenhagen and the Harvard Fatigue Laboratory in Boston. The rich literature of this period describes for the first time the relative utilisation of carbohydrates and fat during exercise and its dependence upon nutrition [52], the effects of adrenaline and insulin on glycemia during exercise, the lactate kinetics during exercise and the positive effect of glucose supplementation on exercise performance. To illustrate the latter finding, Dill, Edwards and Talbott experimented on a young male 13 kg fox-terrier, called Joe. On one occasion, they reported, Joe could run continuously for 17 hours (132 km distance and a climb of 23 km) without being exhausted, thanks to the continuous supplementation of glucose [75].

1941-1955. In this period, major advances in the understanding of the regulation of muscle sugar uptake come with the development of better experimentally controlled animal models. Goldstein and Levine demonstrate that, in eviscerated nephrectomized dogs, insulin and muscular work cause a decrease in blood levels of glucose, as well as of other non-utilisable sugars, but only of those having the same configuration as glucose on the first three carbon atoms [97]. This leads to various speculations on the nature of hexose transport through cell membranes. The Danish researchers Huycke and Kruhøffer hypothesised that 'such transfer might be imagined as occurring through minute "channels" offering different degrees of steric hindrance to the passage of various substances…depending on their detailed, spatial structure' [143]. They did their experiments on the isolated perfused hindlimb of cats. This method is essentially identical to the method on rats that I have used in this thesis. They were also the first to show that in the recovery period following muscular work, glucose uptake remains elevated for some time[143]. Now, 45 years later, we are still trying to explain the mechanism behind this phenomenon.

1956-1967. The permeability of the muscle membrane to glucose and the regulation of muscle glucose uptake becomes an important field of interest for biochemists and biophysicists in St. Louis, Missouri. Extensive work, mainly performed on isolated frog

muscle and rat diaphragm, resulted in a series of ten publications on tissue permeability, published in the Journal of Biological Chemistry [167;203;209]. The studies introduce the use of radioactive 3-O-methylglucose and 2-deoxyglucose for the study of muscle glucose uptake [167;203]. The findings of saturation-type kinetics and the competition among sugars lead to the assumption that transport across the cell membrane occurs at a limited number of sites on the membrane and that it is carrier- or transporter-mediated. In 1967, Holloszy and Narahara introduced the hypothesis that the enhanced permeability of the sarcolemma to sugar associated with muscle contraction is potentiated by Ca^{++} [136].

1962-1975. The development and application of two new invasive experimental techniques on humans caused a rapid progress in the understanding of human muscle metabolism in the sixties and seventies. Firstly, routine sampling of muscle tissue of healthy humans became possible with the coming of the needle biopsy technique, developed by Bergström and Hultman in Stockholm [21]. Utilisation of muscle glycogen during human exercise and its resynthesis during recovery were intensively studied. Secondly, arterio-venous catheterisation over the leg or the arm of healthy, exercising humans was first used for research purposes by Wahren and Felig and allowed measurements of muscle blood flow and arterio-venous exchange of glucose, fatty acids, oxygen and various other metabolites [270;272]. Experiments combining the needle biopsy technique and the arterio-venous catheterisation have contributed tremendously to the understanding of human muscle metabolism through the recent three decades.

1980-1981. A major breakthrough in the understanding of insulin activation of glucose uptake was achieved when Wardzala and Cushman and Suzuki and Kono simultaneously but independently discovered the phenomenon of glucose transporter translocation (see figure 1). First in fat cells [65;255] and shortly thereafter in muscle cells [278], they could demonstrate an intracellular pool of glucose transporters that can be moved to the surface membrane to become active transporters upon stimulation with insulin. Several years later, other laboratories showed that muscle contractions, like insulin, activate the glucose transport mechanism through a similar translocation process [78;92], but that contractions recruit from a different pool of transporters [56;214] (figure 1).

1982-1985. The glucose transporter recruitment hypothesis evoked a rapid growth in research in the field of muscle glucose uptake regulation. In the years that followed, independent laboratories elucidated that insulin and contractions represent two essentially different stimuli that can increase glucose transport independent of each other [205;212] and

additively to each other [70]. Furthermore, contractions seem to have both an acute and a chronic effect on muscle glucose uptake. Richter and co-workers showed, first in rats [227] and later in humans [232], that the insulin sensitivity of skeletal muscle to glucose transport stimulation is increased in the period following a single bout of exercise.

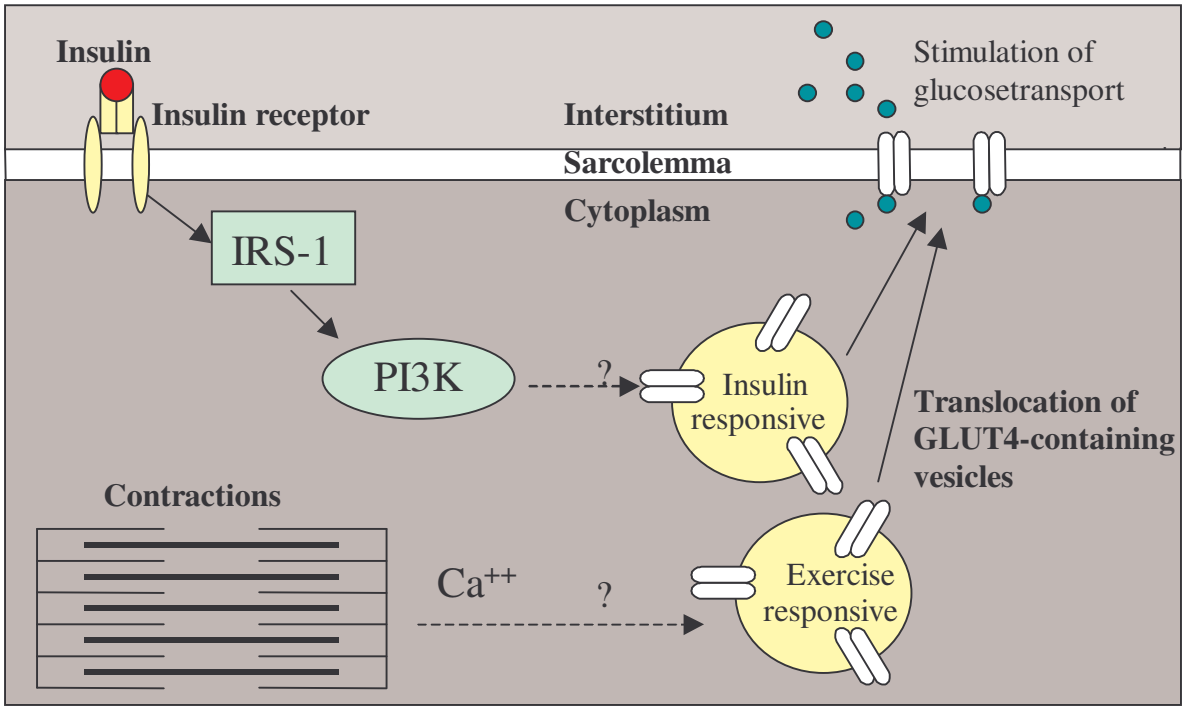

Figure 1: Schematic picture of the process of GLUT4 translocation in a muscle cell and its stimulation by insulin and contractions. Insulin receptor substrate-1 (IRS-1) and phosphatidylinositol-3 kinase (PI3K) are proteins involved in insulin signalling to glucose transport.

1985-1990. This period is characterised by the discovery and cloning of the GLUT family, a family of glucose transporter proteins (GLUT proteins) that are all broadly alike in structure and function (see table 1). The five different members of the family are called isoforms and are numbered in order of their discovery [104;188]. GLUT1, first cloned in 1985 [199], is thought to be the ubiquitous isoform being present in all cell types at low concentrations (except in the blood-brain barrier, where it is the dominant transporter) and serving the role of transporting glucose at basal rates (i.e. when cells are in a comparatively inactive state). GLUT2 and GLUT3 were cloned in 1988 and are predominantly expressed in liver and ß-cells and in the brain, respectively [162;260]. In 1989, James and co-workers finally discovered and cloned the insulin-regulatable glucose transporter GLUT4, eight years after the discovery of its remarkable ability to move back and forth between an intracellular pool and the surface membrane in response to insulin and contractions [149;150]. GLUT4 is

mainly expressed in skeletal and heart muscle and in adipocytes. In 1990, GLUT5 was characterised as a fructose transporter mainly present and active in the small intestines, but also in skeletal muscle [68]. The (pseudo)gene of GLUT6 does not encode a functional glucose transporter protein [161] and the discoverers of the GLUT7 gene, first hypothesised to encode an endoplasmic reticulum protein in hepatocytes [269], have recently admitted that this was a cloning artefact [40]. However, the observation that GLUT4-deficient mouse muscle displays normal insulin-stimulated glucose transport, which cannot be attributed to compensatory action of the other GLUTs, suggests that a still unknown insulin sensitive glucose transporter awaits discovery in skeletal muscle. Interestingly, in a recent abstract, a GLUT4-like gene was cloned (GLUT8), which might be present in human skeletal muscle [237]. All these GLUTs contain ~500 amino acids including 12 membrane-spanning segments that form a channel. The transporters operate by *facilitated diffusion*, which means, allowing transport of sugars down their concentration gradient through this channel without energy-utilising processes. The transporter is hypothesised to continuously alternate between an outward to an inward facing conformation [208]. Apart from the GLUT family, there exist other glucose transporter in the human body, the Na^+-glucose cotransporters, which can transport glucose against its concentration gradient on the expense of ATP utilisation [124;244].

Table 1: Characteristics of the members of the facilitative glucose transporter family

Transporter isoform	**Tissue distribution**	**Characteristics**
GLUT1	Widely expressed. Highly expressed in brain and erythrocytes	Basal glucose transport high affinity to glucose (low K_m)
GLUT2	Liver, kidney, small intestines, ß-cells	Couples pancreatic insulin secretion to glycaemia. High K_m
GLUT3	Neurons, placenta	Low Km. Basal neuronal glucose uptake
GLUT4	Skeletal and cardiac muscle, adipose tissue	Translocatable. Insulin- and exercise responsive transporter. Intermediate K_m
GLUT5	Kidney, small intestine, skeletal muscle	Fructose transporter. Low glucose affinity

1990-2000. The nineties and probably also a great part of the 21st century are and will be characterised by the search for 'signal transduction pathways'. Now the structure of the

muscle glucose transporter and the main activation mechanism (translocation) are known, the big question remains as to how the GLUT4 proteins are actually triggered to move to the sarcolemma in response to insulin and contractions. In the case of insulin, the transduction of the signal between the initial binding of insulin with its receptor and the final translocation of GLUT4 was a big black box, which became a bit smaller with the discovery of a tyrosine phosphorylation cascade downstream of the insulin receptor, involving IRS (insulin receptor substrate) and PI3K (phosphatidylinositol-3 kinase), as depicted in figure 1. The recent development of molecular biology techniques, such as transgenic mice models, provide powerful tools facilitating the search for new proteins involved in insulin signalling. In the case of contractions, the signalling pathway is even a bigger mystery because even the starting point of the signal is poorly understood. The nineties were also characterised by the discovery of some of the processes and proteins involved in the actual mobilisation and trafficking processes of GLUT4, such as ‘budding’ and ‘SNAREing’ (reviewed in [119;221]).

CHAPTER 2: REGULATION OF MUSCLE GLUCOSE UTILISATION DURING EXERCISE

The regulation of carbohydrate utilisation in working muscle is a rapidly growing research field, as judged by the exponential increase in the annual amount of articles published on this topic (see figure 2). The growing interest comes from researchers with a variety of backgrounds, including fundamental researchers (e.g. physiologists, cell biologists) as well as sport scientists (ergogenic effect of carbohydrate supplementation on exercise performance) and endocrinologists (the therapeutical effect of exercise on insulin sensitivity in diabetics).

Figure 2: Annual number of Medline articles containing the keywords 'exercise-skeletal-muscle-glucose-metabolism', relative to the total number of articles published per year. *E.g.*, in 1981, 4 out of 270.000 biomedical articles contained these keywords, whereas in 1996 this ratio was 61 out of 405.000 articles.

Glucose utilisation during exercise: sites of regulation

In muscles, the energy for contractions originates from ATP hydrolysis. Repletion of ATP stores can occur through the anaerobic metabolism of phosphocreatine and glucose and the aerobic metabolism of glucose, fatty acids and amino acids. In sustained intensive exercise bouts, glucose is the principal fuel. Therefore, in the transition from rest to exercise, the

muscle is confronted with a dramatically increased need for glucose. This glucose can be derived intracellularly from glycogen degradation as well as from the uptake of blood-borne glucose. During prolonged strenuous exercise, both processes will take place simultaneously and will complement and possibly regulate each other (reviewed in [118]). The glycogen depots in the skeletal musculature are limited and glycogen depletion can cause fatigue [99;120;249]. The depots of glucose in the bloodstream are also limited, but the blood glucose level is held relatively constant during exercise through increased hepatic glucose output [169]. Liver glycogen depletion can be avoided or delayed by exogenous carbohydrate ingestion [152]. This Chapter will focus on the regulation of glucose utilisation/uptake in muscle. This topic has also been extensively reviewed elsewhere [138;225].

The rate of glucose utilisation in muscle is the product of three processes, i) glucose delivery rate, ii) glucose transport[2] rate and iii) glucose metabolism rate. Upon initiation of exercise, at least the former two processes are activated and all three can be the rate-controlling step in glucose utilisation ([110], reviewed in [225] and [279]). Firstly, the *glucose delivery rate* is dependent on the arterial glucose concentration and the blood flow. At the onset of exercise, blood flow will increase 10-fold or more [4] and also arterial glucose concentration may increase under some circumstances [234]. With the aid of the recently developed microdialysis technique to assess interstitial metabolite concentrations in human muscle during dynamic exercise, interstitial glucose concentrations have been shown to increase from 3.7 mM at rest to 5.3-5.6 mM during exercise of increasing intensities [192]. This indicates that glucose delivery is enhanced and probably not rate-limiting during exercise. Secondly, the *glucose transport rate* across the sarcolemma is dependent on the number of glucose transporters (GLUT4) in the membrane, possibly the intrinsic activity of GLUT4 and the transsarcolemmal glucose concentration gradient. During exercise there is a several-fold increase in the amount of GLUT4 on the cell surface membrane, as shown in rats [190] and in humans [177;259]. The regulation of exercise-induced glucose transport

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[2] In this thesis, the terms *glucose uptake* and *glucose transport* are not used as synonyms. *Glucose uptake* is the extraction of glucose from the blood by the limb (consisting primarily but not exclusively of skeletal muscle cells) and refers methodologically to the measurement of arterio-venous differences, multiplied by flow (= leg or hindlimb glucose exchange). *Glucose transport* is the actual transsarcolemmal uptake of glucose into the skeletal muscle cells and refers methodologically to the uptake of radiolabelled glucose analogues by muscle tissue, corrected for extracellular space.

stimulation and GLUT4 translocation will be discussed in detail below. Thirdly, once glucose is taken up by the cell, the *glucose metabolism* starts with phosphorylation to glucose-6-phosphate (G6P)[3] by hexokinase to enter the glycolysis pathway (or glycogen synthesis). Under some exercise conditions, glucose phosphorylation can be rate-limiting, due to the accumulation of G6P from glycogen breakdown, but in most of the situations, and especially in resting muscles, the glucose transport step is limiting for muscle glucose uptake [225].

Insulin and contractions are two potent and independently effective stimuli for muscle glucose transport [212]. Contractions stimulate glucose transport in isolated muscle preparations [275], thus, (a) local factor(s) (not neuronal or hormonal) must cause GLUT4 translocation during contractions. However, besides the local effect of contractions, other hormonal or neuronal processes can contribute to increased muscle glucose uptake during exercise *in vivo* [134]. Although plasma insulin concentrations may decrease during exercise, the delivery of insulin to the muscle cells increases through increased capillary recruitment. Thus, part of the increased muscle glucose uptake during exercise can be attributed to the action of insulin. This is supported by the finding that exercise-induced glucose uptake stimulation in the total absence of circulating insulin, in insulin-deficient dogs, is reduced [280]. Apart from insulin, also other humoral factors may be involved. ß-endorphin is suggested to be released into the circulation during exercise [45] and is hypothesised to be a stimulator of muscle glucose uptake [86]. Finally, there is conflicting evidence as to whether sympatho-adrenal hormones play a role in muscle glucose uptake regulation during exercise [8;151;224;233].

For a discussion of the mechanism of insulin stimulation of glucose transport, I refer to Chapter 3. Below I will sum up the possible local signalling mechanisms, leading to the acute contraction-induced increase in glucose transport in skeletal muscle cells.

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[3] Abbreviations used in this thesis: AMPK, 5'AMP-activated protein kinase; cAMP, cyclic adenosine monophosphate; G6P, glucose-6-phosphate; GLUT, glucose transporter isoform; GS, glycogen synthase; IRS, insulin receptor substrate; NIDDM, non-insulin-dependent diabetes mellitus; NO, nitric oxide; NOS, nitric oxide synthase; PI3K, phosphatidylinositol-3-kinase; PKB, protein kinase B, PKC, protein kinase C.

Molecular mechanisms involved in GLUT4 translocation in muscle during exercise

As outlined in Chapter 1, contractions, like insulin, cause increased cell permeability to glucose by translocation of GLUT4-containing vesicles to the surface membrane. Formerly, it was believed that only part of the increase in muscle glucose transport was attributable to translocation and that also the 'intrinsic activity' of the GLUT4 transporters was stimulated [166]. Now, many researchers believe that an increase in the amount of GLUT4 on the surface membrane is solely responsible for increased transport capacity, based on studies using exofacial surface labelling of GLUT4 with bis-mannose photolabels, reporting 3- to 5-fold increases in cell surface GLUT4 content as well as glucose transport [190]. However, the literature reports a wide variety in the fold-increases in cell surface GLUT4 content in response to exercise/contractions depending on the method of evaluation (reviewed in [123]). Direct visualisation in the electron microscope detects 9- to 29-fold increases [214]. Studies where glucose transporter content in plasma and intracellular membranes were assessed by biochemical fractionation techniques report 2- to 3-fold increases [34;56;78;238]. GLUT4 protein content in sarcolemmal giant vesicles increases approximately 2-fold with exercise [177;215]. It should be noted that during contractions, GLUT4 vesicles move not only to the outward-facing plasma membrane, but also to the transverse tubules (T-tubules)[4][214;238]. Furthermore, contractions recruit GLUT4 vesicles from a different intracellular pool than insulin [56;79]. Ploug et al. have recently identified this contraction-sensitive pool as transferrin receptor-positive recycling endosomes [214].

Virtually every event that occurs inside a muscle cell in the transition from rest to contractions is a possible candidate for initiating a signal, leading to GLUT4 translocation (reviewed in [123;137;147;225;230;290]). Membrane depolarisation and Ca^{++} -release from the sarcoplasmic reticulum are two events that initiate contractions and actually take place before the contractions of the filaments. Therefore I will call them feed-forward mechanisms, i.e., they are presumably independent of the result, the work output of the contractions. Intracellular Ca^{++} accumulation, but not membrane depolarisation, is thought to be involved in glucose transport stimulation during contractions (table 2). Ca^{++} will even stimulate glucose transport at intracellular concentrations, too low to elicit contractions, illustrating that it is a

[4] T-tubules are invaginations of the sarcolemma, conducting action potentials and substrates deep into the fibre.

feed-forward mechanism [295]. Jóhannsson et al. [154] have demonstrated that in slow-twitch[5] but not fast-twitch muscles, the glucose transport rate during electrical stimulation in rats was closely related to the stimulation frequency and thereby the cytosolic Ca^{++} concentration. Further downstream Ca^{++}-dependent signalling leading to GLUT4 translocation is not known but the involvement of the Ca^{++}-dependent protein kinases, protein kinase C (PKC)[6] and calmodulin-dependent protein kinase II, is hypothesised (table 2). Khayat et al. and Kawano et al. have recently demonstrated that insulin-independent, Ca^{++}-dependent glucose transport stimulation is mediated by translocation and activation of the conventional PKCβ_2 [160;163]. Richter et al. have previously shown that PKC is translocated in response to muscle contractions [226]. Besides possible mediation through PKC, Ca^{++} may also affect muscle glucose transport rate by activating Ca^{++}-dependent nitric oxide synthase (NOS) and nitric oxide (NO) release (see [11] and below).

Kinins, nitric oxide (NO) and adenosine are local autocrine/paracrine factors that are produced in skeletal muscle at work. A direct role for the kallikrein-kinin system in the modulation of contraction-induced glucose transport in muscle is both supported and contradicted (table 2; reviewed in [195]).

It is agreed upon that acute exercise increases NO release and NO synthase activity [12;235] and that NO can stimulate glucose uptake in muscle (reviewed in [11]). However, a role for NO in contraction-induced glucose transport is not fully supported (table 2). Whereas a recent study on humans reported that NO synthesis inhibition by L-NMMA infusion during exercise reduces muscle glucose uptake by upto 48%, without affecting hemodynamics [29], this is contradicted by others using L-NAME (Frandsen, Hellsten, Bangsbo & Saltin, unpublished results). One reason for this discrepancy may be that in humans, the allowed doses of the inhibitors are too low to completely inhibit NO production in skeletal muscle cells. But also in *in vitro* studies, where higher doses of inhibitors can be used, reduced contraction-induced glucose transport by NOS inhibition has not consistently been found (table 2).

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[5] Skeletal muscle fibres are divided into slow-twitch, fast-twitch oxidative and fast-twitch glycolytic fibres, based on their contractile and metabolic characteristics.

[6] There are many PKC isoforms, subdivided in three subfamilies: conventional PKCs (cPKC: α, β_1, β_2, γ), novel PKCs (nPKC: δ, ε, η, θ) and atypical PKCs (aPKC: λ, ζ).

	Supportive	Non-supportive
Feed-forward mechanisms		
Membrane depolarisation		[136;139]
Ca^{++}	[136;139;295;299]	
Calmodulin	[37;247]	[106;144]
Protein kinase C	[54;129;144;160;163;296]	
Autocrine mechanisms		
Kallikrein-kinin	[74;168]	[59;250]
Nitric oxide	[13;29;236;236;298]	[85;156]
Adenosine	[111;218;266]	
Metabolic feed-back mechanisms		
Glycogen depletion	[91;98;131;159;231]	[146] [283]
AMP-activated protein kinase	[20;121;122;146;179;197]	
Other signalling intermediates		
Insulin receptor substrate 1/2		[135;176;288]
Mitogen-activated protein kinases		[123;289]
Phosphoinositide-3 kinase	[288]	[176;185;190;288;293]
Protein kinase B	[259;262]	[35;191]

Table 2: Studies providing evidence for or against the involvement of various molecules or processes in contraction-stimulated glucose transport in skeletal muscle. For explanation, see text.

Adenosine is produced in the interstitium by AMP degradation and released by contracting muscle cells into the interstitium [125;126]. Interstitial adenosine affects both the glucose delivery step, through its vasodilator effect on vascular muscle, as well as the glucose transport step, by autocrine binding with adenosine receptors at the sarcolemma of skeletal muscle cells (reviewed in [132]). Different adenosine receptor subtypes (A_1, A_{2a}, A_{2b} and A_3) have been identified and are co-expressed in various body tissues. Which receptor subtypes are expressed in skeletal muscle is not well understood. A RT-PCR study has reported that skeletal muscle is devoid of A_1 and A_3 receptor mRNA, but expresses high and low levels of respectively A_{2a} and A_{2b} receptor transcripts [76]. In contrast, studies with specific antibodies or antagonists report the presence of A_1 and/or A_2 receptors in skeletal muscle tissue ([48;266;267]; T. Graham, personal communication; J. Lynge & Y. Hellsten, personal communication).

Selective (A_1) or non-selective adenosine receptor antagonism has been shown to reduce glucose uptake in exercising human and rat muscle in the presence [218;266] or in the

absence of insulin [111]. Agonism of adenosine receptors, however, does not seem to increase glucose transport in resting or contracting muscles [266]. Furthermore, some researchers believe that adenosine regulation of glucose transport is restricted to slow-twitch fibres [187;266], whereas other do not [111]. Therefore, the role of adenosine in the regulation of muscle glucose uptake remains controversial.

As judged by the presently available literature, stimulation of glucose transport in contracting muscles may require the action of multiple autocrine/paracrine factors, although the magnitude of effect is generally rather limited and the evidence sometimes conflicting.

Jacob Ihlemann et al. [146] have recently published intriguing data, where incubated soleus muscles were electrically stimulated with varying tensions and, hence, metabolic rates, while stimulation frequency was held constant. Their findings clearly illustrate that the stimulation frequency, an important determinant of intracellular Ca^{++} concentration, is not the only determinant of contraction-induced glucose transport, but that the force development and the metabolic stress in the muscle largely co-vary with the magnitude of glucose transport stimulation [146]. The magnitude of glucose uptake in contracting muscles has previously been related to the metabolic state of the muscle [274].

One of the first evidences for the existence of a metabolic feedback mechanism in the exercise regulation of muscle glucose uptake came from Gollnick et al. [98]. During 2-legged cycling, glucose uptake was higher in the leg that had previously been depleted from glycogen during one-legged exercise, compared to the control leg. This was later expanded by Hespel & Richter, demonstrating a negative correlation between muscle glycogen content and glucose uptake in perfused, contracting rat hindlimbs [131]. They ascribed part of this phenomenon to a direct effect of glycogen on the glucose transport step and part of it to the inhibition of hexokinase (glucose phosphorylation) and accumulation of G6P in contracting muscles with high glycogen levels. So it can be hypothesised that glycogen plays a regulatory role in the mobilisation of GLUT4 during contractions, similar to the potential role it plays in hypoxia-stimulated glucose transport, as suggested by Reynolds et al. [222]. If this appears to be the case, glycogen's regulatory action could be either direct or indirect. A direct mechanism could involve a structural linkage between glycogen particles and GLUT4-containing vesicles [57]. An indirect mechanism could involve a sensor, probably a protein, which can sense/monitor the intracellular glucose/glycogen availability and initiate a signal pathway to correct the glucose uptake of the cell accordingly. AMP-activated protein kinase (AMPK) is a such a fuel gauge, which has been shown to activate energy-generating

processes in response to decreased intracellular energy status and substrate availability [285]. Interestingly, AMPK, which is activated during contractions, is now suggested to be involved in contraction signalling leading to muscle glucose transport (see Table 2), based on the observation that stimulation of AMPK with AICAR increases glucose transport in an insulin-independent manner [121;179;197]. The possible role of glycogen in glucose transport stimulation, the underlying mechanism and the involvement of AMPK in contraction signalling to glucose transport, are extensively investigated in the experiments of this thesis (described in Chapter 4).

Insulin exerts its stimulating effect on glucose transport through a complex intracellular signalling cascade (see Chapter 3). The proteins involved in this cascade are potentially also involved in the contraction signal leading to increased glucose uptake, since both signals eventually converge in the process of mobilisation of GLUT4-containing vesicles to the sarcolemma. However, as shown in table 2, almost all the studies in rats and humans have failed to report activation of these insulin signalling intermediates with exercise. Additionally, inhibition of phosphoinositide-3 kinase (PI3K) by wortmannin at doses that inhibit insulin-induced glucose transport, does not lead to decreased contraction-induced glucose transport [288]. Thus, the presently identified signalling proteins leading to GLUT4 translocation in response to insulin are not involved in contraction signalling. This emphasises the complete different nature of insulin and contractions as stimuli of muscle glucose transport.

In conclusion, the contraction stimulus leading to increased glucose transport in muscle is a concurrence of processes. A rise in cytosolic Ca^{++} concentration, the release of autocrine/paracrine factors and metabolic feedback from fuel depletion are possible initiators of the signal. Protein kinases, like PKC and AMPK, are supposedly involved in the transduction of the signal to the endpoint, *i.e.* GLUT4 translocation.

Although purely hypothetical, figure 3 indicates possible modes of interaction and points of convergence for the various proposed mediators of contraction-induced glucose transport. The central initiators of the signal would be Ca^{++} and AMP. The AMP signal could possibly interact with the Ca^{++} signal at the level of NOS. There is some recent evidence that AMPK can activate NOS through phosphorylation in the presence of Ca^{++}-calmodulin [51]. Similarly, NOS has also been proposed to be a substrate for PKC [201]. However, firm evidence for the regulation of NOS by AMPK or PKC in skeletal muscle is lacking. Another possible site of interaction of autocrine factors may be situated at the level of the ‘second messengers’, cyclic AMP (cAMP) and cyclic GMP (cGMP) (figure 3). Both NO and adenosine to some extent

exert their action through these second messengers and the involvement of their regulatory enzymes (guanylate/adenylate cyclases, phosphodiesterases) in the regulation of glucose transport are worthwhile investigating [297]. As to the interaction of adenosine with other contraction-activated pathways, A_1-adenosine receptor agonism in rat adipocytes has recently been shown to induce translocation of PKC and GLUT4 [180]. This would then indicate that PKC is a downstream signalling intermediate of adenosine action on glucose uptake in contracting muscle.

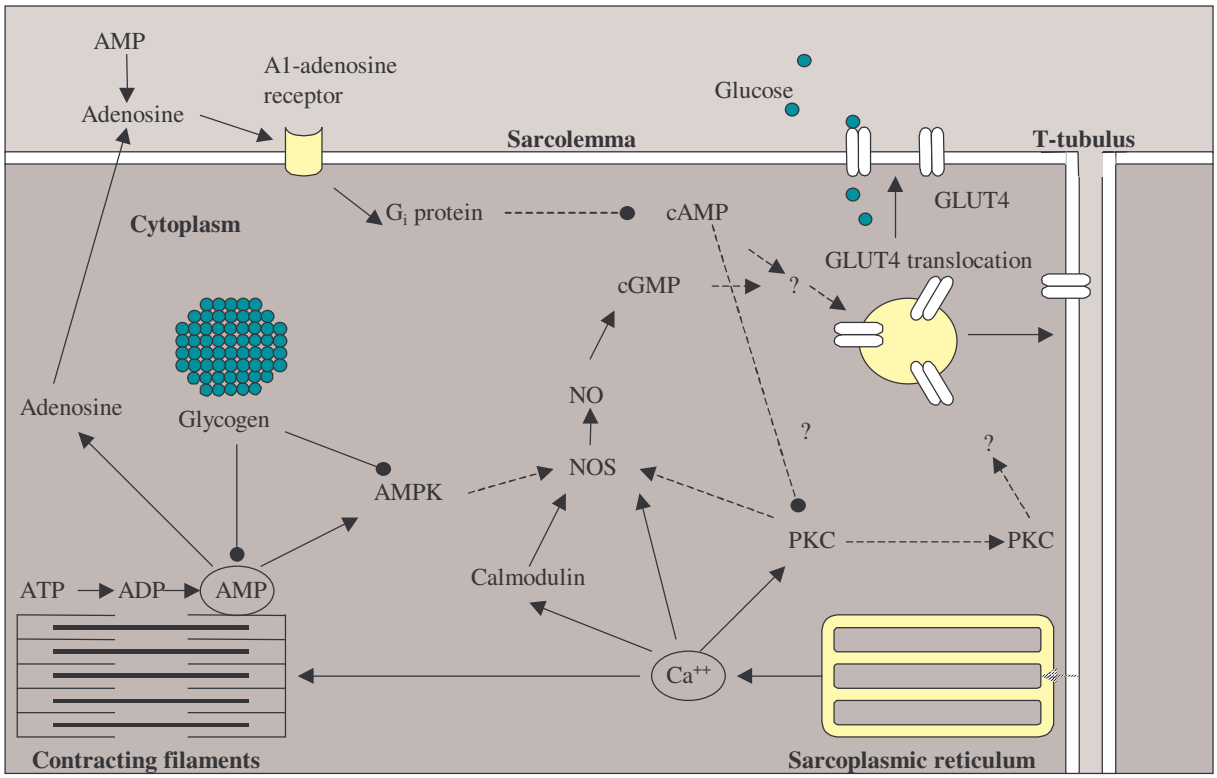

Figure 3: Hypothetical model of interaction of feed-forward, feed-back and auto/paracrine factors in the regulation of GLUT4 translocation during contractions in a muscle cell. Sharp and round arrowheads indicate activation and inhibition, respectively. Dotted lines are hypothetical assumptions based on few experimental evidence. The starting points are indicated with circles: a rise in the cytosolic concentration of Ca^{++} and AMP.

Although all of the above possible interactions and mechanisms are valuable in designing and directing future research, their character is highly hypothetical, since their validity has not been tested in skeletal muscle yet. It is clear that we are still at the beginning of a long way leading to complete understanding of contraction-induced glucose transport in skeletal muscle.

The role of hypoxia in contraction-stimulated glucose transport

Although it has long been established that hypoxia is a potent stimulus of *in vitro* muscle glucose uptake [209;220], it is not known whether local tissue hypoxia participates in exercise-stimulated muscle glucose uptake. Reducing the oxygen content in the inhaled air during exercise leads to enhanced muscle glucose uptake compared to normoxia [33;60;158;170], whereas the inhalation of a hyperoxic gas mixture does not seem to reduce exercise-induced muscle glucose uptake [282]. However, these studies are hard to interpret, because alterations in the oxygen content of the inhaled air have only a minor effect on muscle oxygen extraction and availability [282;304]. More direct estimations of the mitochondrial redox state of human skeletal muscle during exercise may therefore provide a better insight. Some of these studies have led to the conclusion that the redox state rises during exercise, even at intensities that cause lactate accumulation [304], whereas others have observed an exercise-induced decrease in redox state [130;241;282]. Thus, it remains to be established whether local hypoxia is a participant in stimulating muscle glucose uptake.

Since the results from an *in vitro* study, showing that the maximal stimulating effects of hypoxia and contractions were not additive, have been published by an influential research group [46], it has become a dogma that hypoxia and contractions are identical stimuli for muscle glucose transport. Based on this observation, these and other researchers have used the easily applicable hypoxia stimulus as a surrogate for contractions in their *in vitro* studies on muscle glucose transport regulation. Consequently, studies that make conclusions on contraction stimulation of glucose transport, whereas they have only studied hypoxia, are numerous [9;36;37;115;222;223;294;303]. However, the number of studies that have systematically investigated and compared the nature of hypoxia and contractions as stimuli of glucose transport is very limited. Therefore, it became one of the main objectives in this thesis (See Chapter 4).

The mechanism by which hypoxia stimulates muscle glucose uptake is not fully understood (reviewed in [137;301]). Hypoxia, like contractions, induces a translocation of GLUT4-containing vesicles to the plasma membrane [46;223]. In support of this notion, hypoxia does not stimulate muscle glucose transport in transgenic GLUT4-deficient mice [303]. Hypoxia is believed to induce GLUT4 translocation through a Ca^{++}-dependent mechanism [46;294]. Furthermore, regulatory roles of glycogen [222] and AMPK (Laurie Goodyear, Joslin Diabetes Center, Boston; personal communication) on hypoxia stimulation of muscle glucose transport have been proposed. In conclusion, most literature data indicate

that hypoxia and contractions stimulate muscle glucose uptake by identical cellular mechanisms. This does, however, not translate to the *in vivo* situation, because tissue hypoxia is probably not an important contributor to increased glucose uptake in active muscle.

CHAPTER 3: MECHANISMS OF INCREASED INSULIN SENSITIVITY IN MUSCLE FOLLOWING EXERCISE

'Living systems are worn out by inactivity and developed by use'

(A. Szent-Györgyi)

Insulin action in skeletal muscle

The skeletal musculature, representing 40% of the human body mass, is the most important site for insulin-mediated whole body glucose disposal and is therefore a key player in the maintenance of euglycaemia [18]. Insulin-induced glucose transport, the rate-controlling step in glucose uptake in muscle [55;155], is regulated by the insulin-sensitive glucose transporter GLUT4 [149]. Similar to the exercise-induced mechanism, activation of GLUT4 by insulin is accomplished by translocation of GLUT4-containing vesicles from intracellular domains to the plasma membrane and T-tubules [65;77;108;281]. The signal pathway leading to this mobilisation process is not fully understood. However, unlike contractions, the starting point of signal transduction is well described and involves the binding of insulin with its own receptor on the sarcolemma [157]. Below, I will briefly summarise the present knowledge on insulin signalling in metabolic events. For more detailed information on this topic, I refer to up-to-date, comprehensive reviews [2;140;248;257].

Insulin regulates a wide spectrum of metabolic and growth processes, including glycogen, lipid and protein synthesis, antilipolysis, gene transcription, growth, differentiation and glucose uptake [257]. Therefore, diverse signalling pathways diverge from the insulin receptor to generate these specific endpoint responses. The unravelling of this web of signal transduction is as complex as it is hot (~10 articles per week!). Several proteins are now believed to be intermediates of the insulin pathway leading to increased glucose transport in muscle cells: insulin receptor substrate (IRS), phosphoinositide-3 kinase (PI3K), protein kinase B (PKB) and atypical protein kinase C (aPKC) (see figure 4).

The intracellular portion of the insulin receptor contains a tyrosine protein kinase [157]. Insulin binding activates this kinase, leading to autophosphorylation and tyrosine phosphorylation of IRS-1and IRS-2 [284]. Subsequent binding and activation of IRS with PI3K is essential for insulin stimulation of muscle glucose transport [185;190;288]. PI3K is a lipid kinase and phosphorylation of phosphatidylinositol induces further downstream activation of serine-threonine protein kinases [186]. Protein kinase B has been identified as a downstream effector of PI3K [41;128;173]. Also

some of the atypical isoforms of PKC are believed to be activated by insulin in a PI3K-dependent manner [30;253], but an agreement about which isoforms are involved in glucose transport stimulation, is still 'in construction'. At the moment it seems like both PKBα and β [43];[263;276] [109]and the atypical PKCs, ζ and λ [14;174] are involved in glucose transport stimulation. The kinases PKB and PKC are believed to be activated through phosphorylation by a phosphoinositide-dependent kinase (PDK) [1;15;184;273]. As the name explains, this kinase is activated and its action is facilitated by the phosphorylated lipid products (PIP3) of the PI3K reaction (see figure 4) [64]. Insulin activation of PKB is also believed to involve translocation to the plasma membrane [5;102].

The insulin signalling cascade leading to glucose transport and glycogen synthase activation (GS) is probably common down to the level of PKB. Further downstream signalling is believed to be specific for GS and involves glycogen synthase kinase-3 (GSK3) [252]. Phosphorylation of GSK3 by PKB causes deactivation of this kinase, which can inhibit GS by phosphorylation [63;264]. In contrast to the GS activation, the signalling downstream of PKB, leading to increased glucose transport has not yet been established (figure 4). The observation that PKB is associated with GLUT4-containing vesicles, which is enhanced by insulin stimulation, may prove important in this process [43].

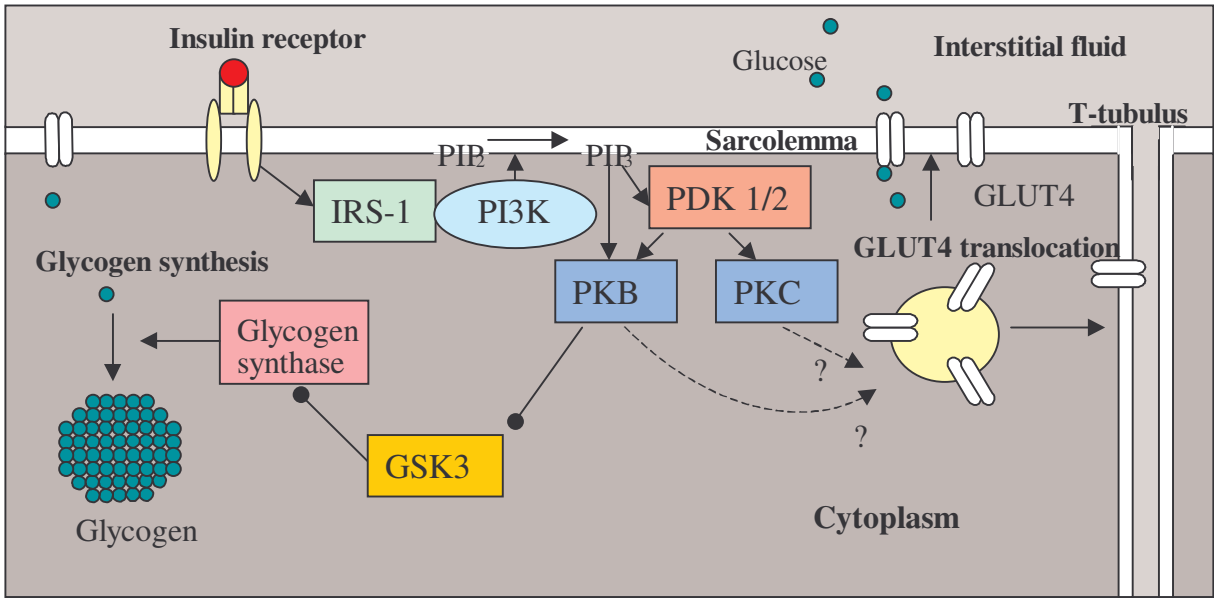

Figure 4: Insulin signalling pathway leading to stimulation of glucose uptake (GLUT4 translocation) and glycogen synthesis. Dotted lines are missing links. Sharp arrowheads indicate activation through phosphorylation, binding or translocation. Round arrowheads indicate inhibition through phosphorylation.

This is as far as our understanding reaches today. It is not known how many more proteins need to be identified before we can get a clear understanding of insulin signalling. It is frustrating to note that many of these signalling intermediates can also be activated by other growth factors without concomitant effects on metabolism. So it looks as if we are still missing an important 'clue'. The crosstalk with other signalling pathways and the temporal (time-course of activation) and spatial (localisation of proteins) aspects of signalling activation may prove very important for our understanding in insulin signalling [80].

Increased post-exercise insulin action on glucose transport: a role for glycogen depletion?

Non-insulin-dependent diabetes mellitus (NIDDM[7]) patients have too high fasting blood glucose levels, resulting from an imbalance between insulin sensitivity and insulin secretion [69]. Impaired insulin sensitivity to glucose uptake in peripheral tissues, primarily muscle, is the earliest abnormality and presumably responsible for the aetiology of the disease [69]. Physical exercise has beneficial effects in the treatment and prevention of many diseases [95], including NIDDM (reviewed in [83;127;211])[141;204]. The therapeutic effect in NIDDM has been advocated to increased glucose utilisation during and after exercise [268;271]. In 1982, Richter et al. observed in rats that in the period lasting several hours following an exercise bout, skeletal muscle exhibits increased insulin sensitivity[8] to glucose uptake [227]. This observation was later expanded to humans and patients with insulin resistance, such as NIDDM [73;232]. Thus, increased post-exercise muscle insulin sensitivity may prove an important factor in the improved glucose control in individuals with NIDDM [246] and in the preventive effect of regular exercise on the development of NIDDM (reviewed in [101]). An insight in the molecular mechanism of the phenomenon may therefore facilitate the rational development of drugs in a treatment for the world's 150 million patients with NIDDM [292].

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[7] The disease diabetes mellitus is divided into type-I (insulin-dependent diabetes mellitus, IDDM; a deficiency in insulin production) and type-II (non-insulin-dependent diabetes mellitus, NIDDM; peripheral insulin resistance and inadequate compensation) diabetes

[8] Increased insulin sensitivity is defined as a shift in the dose-response curve to the left, with a decrease in the insulin concentration required to cause a halfmaximal effect. Increased insulin responsiveness refers to an increase in the maximal attainable effect of insulin.

In order to understand how prior exercise promotes insulin's ability to stimulate glucose uptake in muscle, it is important to define the site of control. Using the one-leg exercise model, Richter et al. identified that increased insulin sensitivity is restricted to the exercised leg, pointing to a local mechanism [232]. Several studies have shown that exercise does not stimulate the insulin binding to its receptor [26;27;261;305] nor the insulin receptor tyrosine kinase (IRTK) activity [261;287]. Insulin stimulation of PI3K activity has both been shown to be increased [302] as well as decreased [100;287] following exercise. Similarly, insulin stimulation of PKB in human muscle is reported to be increased [259] as well as unchanged [286] after exercise. Thus, exercise-induced enhancement of insulin-stimulated GLUT4 translocation in muscle does not involve the early steps, but possibly the more downstream steps of the insulin signalling pathway. In agreement, also other situations of increased insulin sensitivity, such as after calorie restriction, occur independent of the initial insulin signalling steps [94].

The period following intense exercise is characterised by depleted glycogen stores in muscle. Consequently, upon the first observation of increased post-exercise insulin sensitivity, glycogen depletion was looked upon as a possible regulator of insulin action in muscle [24;87;227]. A causal role for glycogen depletion in this process is contradicted by the fact that increased insulin sensitivity in exercised muscles is persistent in the absence of glycogen depletion [93;227;306]. On the other hand, increased post-exercise insulin action is reversed when carbohydrates are fed and glycogen stores replete, but not when fat is fed and glycogen stores remain depleted [47]. Furthermore, exposure of the exercised muscle to a glucose analogue (2-deoxy-glucose) which can be taken up and phosphorylated but not converted into glycogen, does not lead to reversal of enhanced insulin action [107]. Glycogen depletion induced by fasting or epinephrine infusion, similar to exercise, enhances muscle insulin action to glucose transport [151;207]. Finally, increasing muscle glycogen levels above normal (glycogen supercompensation) leads to decreased insulin-stimulated glucose uptake in rats [229] and humans [113]. Thus, most but not all of the evidence suggests a regulatory role of glycogen on muscle insulin action, possibly accounting for increased insulin sensitivity following exercise.

Increased post-exercise insulin action on glycogen synthase

In a classical experiment, Bergström and Hultman demonstrated that a glycogen-depleting exercise bout is followed by a period of rapid repletion of glycogen stores to levels exceeding pre-exercise values (*i.e.* glycogen supercompensation), provided a high-carbohydrate diet is ingested

[22]. Sitting on either side of a bicycle, they performed an exercise bout with only one leg, leaving the other rested. Enhanced glycogen synthesis and glycogen supercompensation only occurred in the exercised leg, pointing to a mechanism located in the muscle cells [22]. From the previous paragraph, it is evident that enhanced insulin sensitivity to muscle glucose transport may be an important factor in the enhanced post-exercise glucose utilisation, directed to glycogen synthesis [93]. Muscle glycogen synthase (GS), a rate-controlling enzyme in glycogenesis, is activated by insulin (reviewed in [58;112;183;217]). Prior exercise is known to increase the insulin sensitivity to muscle glycogen synthase activation [24;227]. Parallel to its role in the regulation of glucose transport, glycogen depletion may in part also be responsible for the observed exercise effects on insulin-activated glycogen synthase. Numerous studies have observed a negative correlation between the muscle glycogen content and glycogen synthesis rate/GS activity in basal and insulin-stimulated muscles [24;67;91;200;229]. Although the existence of a product inhibition (negative feed-back) by glycogen on processes that lead to its synthesis makes teleological sense, these correlations do not necessarily prove causality. Since few studies have addressed or investigated the mechanism by which glycogen operates upon insulin activation of glucose transport and glycogen synthesis, we have designed experiments to investigate this (See Chapter 4).

CHAPTER 4: EXPERIMENTS

'Problems worthy of attack
prove their worth by hitting back'
(Piet Hein, 1905-1996)

From the introductory chapters it became evident that many aspects of the regulation of muscle glucose uptake and insulin action by exercise are poorly understood. Therefore we have conducted a series of studies in an attempt to elucidate the signalling molecules and pathways involved in contraction- and hypoxia stimulation of glucose transport and in increased insulin action following exercise. The experiments, described in this thesis are performed at the Laboratory of Exercise Physiology (Prof. dr. P. Hespel), Catholic University Leuven, and the Laboratory of Human Physiology (Prof. dr. E.A. Richter), August Krogh Institute, University of Copenhagen, Denmark.

Methods and aims

For the study of the regulation of muscle glucose metabolism, the isolated muscle incubation and the hindlimb perfusion are the most frequently used *in vitro* animal (rat) models (critically compared and reviewed in [25]). We have mainly used the hindlimb perfusion, which is very suitable for the measurement of muscle glucose uptake (glucose exchange) and glucose transport rate (uptake of radioactive glucose analogues) [291]. This *in situ* model allows perfusate supply through an intact vasculature as well as electrical stimulation of the sciatic nerve to induce contractions. For the assessment of cell surface membrane GLUT4 content with surface label compounds [53;189], we have used the isolated muscle incubation model. Muscles, perfused or incubated, were exposed to insulin or subjected to electrically stimulated contractions or decreased perfusate oxygen content (hypoxia) . Following stimulation, muscle samples were quickly frozen and determined for various metabolite concentrations and enzymatic activities, using *in vitro* activity assays. Experimental groups were established by the addition of various chemical compounds (inhibitors/antagonists) during hindlimb perfusion or by altering pre-experimental muscle glycogen levels by a preconditioning protocol including swimming exercise and diet. Whereas in the human skeletal musculature, individual muscles are always a mixture of fast- and slow-twitch fibre types [153], rats have some individual muscles with almost exclusively either fast-twitch (in the white portion of the gastrocnemius ~100%) or slow-twitch (in soleus ~85%) fibres

[6;7;72]. This unique feature allowed us to study the proper metabolic characteristics of the different fibre types.

Another advantage of the perfused hindlimb model is that the hindlimb microvasculature is highly dilated, due to the lack of sympathetic tone. Therefore, the vasodilative effect of some interventions, such as hypoxia or insulin stimulation, is probably excluded. This allows us to focus mainly on muscle cell surface membrane permeability to glucose, rather than a combination of glucose availability and membrane permeability. Furthermore, most [103;290;291], but not all [133] evidence indicates that perfusion flow/metabolite delivery is not rate-limiting for metabolite uptake in the perfused rat hindlimb model.

The overall objectives of the experiments were to examine the regulation of exercise-stimulated glucose uptake and insulin action in mammalian skeletal muscle and to elucidate signalling mechanisms involved in metabolic effects of muscle contractions. Paper I & II tried to identify the similarities or dissimilarities of hypoxia and contractions as stimuli of muscle glucose uptake. Paper III, IV and V (and preliminary work) were designed to explore underlying mechanisms of the regulatory role of glycogen in contraction and insulin effects on muscle glucose transport. The specific aims of each experiment were:

Paper I: To compare the effects of selective A_1-adenosine receptor antagonism on glucose uptake in contracting and hypoxic skeletal muscle in the presence or absence of insulin. To investigate additivity of hypoxia and contractions as physiological stimuli of muscle glucose uptake.

Paper II: To investigate the effects of wortmannin and calphostin C, inhibitors of protein kinase C, on muscle glucose uptake and transport, when stimulated by contractions or hypoxia. To investigate the potency and additivity of the maximal effects of contractions and hypoxia as stimuli of muscle glucose uptake.

Paper III: To study whether cell surface GLUT4 content in contracting muscle is dependent on the pre-contraction muscle glycogen content. To elucidate whether the persistent increased glucose transport rate in muscle after exercise, when fed a fat-rich diet, is due to persistent GLUT4 transporters at the muscle surface membrane.

Paper IV: To explore the potential role of AMP-activated protein kinase (AMPK) in the contraction signal leading to increased muscle glucose transport and to investigate whether AMPK activation is dependent on muscle glycogen content.

Paper V: To study the insulin activation of glucose transport, cell surface GLUT4 content and the activities of known insulin signalling intermediates in muscles with varying glycogen content.

Papers I-V

So far, the experiments have led to five papers. Throughout the general discussion (Chapter 5), these papers will be referred to by their roman numerals. Reprints, pre-prints and manuscripts of the papers are presented in the Appendix.

I. Wim Derave and Peter Hespel
Role of adenosine in regulating muscle glucose uptake during contractions and hypoxia in rat skeletal muscle.
Published in Journal of Physiology (London) 515 (1): 255-263, 1999.

II. Jørgen F.P. Wojtaszewski, Jan L. Laustsen, Wim Derave, Erik A. Richter
Hypoxia and contractions do not utilize the same signaling mechanism in stimulating skeletal muscle glucose transport
Published in Biochimica et Biophysica Acta 1380: 396-404, 1998.

III. Wim Derave, Sten Lund, Geoffrey D. Holman, Jørgen F.P. Wojtaszewski, Oluf Pedersen and Erik A. Richter
Contraction-stimulated muscle glucose transport and GLUT-4 surface content are dependent on glycogen content
Published in American Journal of Physiology (Endo. Metab.) 277: E1103-E1110, 1999.

IV. Wim Derave, Hua Ai, Jacob Ihlemann, Lee A. Witters, Søren Kristiansen, Erik A. Richter and Thorkil Ploug
Dissociation of AMP-activated protein kinase activation and glucose transport in contracting slow-twitch muscle.
Accepted in Diabetes

V. Wim Derave, Bo Falck Hansen, Sten Lund, Søren Kristiansen and Erik A. Richter
Muscle glycogen content affects insulin-stimulated glucose transport and protein kinase B activity
Submitted to American Journal of Physiology (Endo. Metab.)

Unpublished work

Some of the results of our experiments, relevant to this thesis, await report in a full manuscript. These results are discussed below.

Are GLUT4-containing vesicles structurally linked to glycogen particles?

In Chapter 2 and in Paper III and IV, it was noted that the glycogen content in skeletal muscle cells - either directly or indirectly - sets the rate of glucose entry during contractions. A direct controlling mechanism has been hypothesised and could involve a structural link between glycogen and GLUT4, e.g. GLUT4-containing vesicles are bound to glycogen particles, making them 'inaccessible' for mobilisation to the surface membrane. Evidence for this hypothesis was presented on a symposium, but never published [57]. Therefore, in collaboration with Søren Kristiansen, we have investigated a possible structural link or co-localisation of glycogen and GLUT4 in skeletal muscle with various techniques. The results are summarised below (Kristiansen, Derave & Richter, unpublished observations).

- Glycogen particles from rat skeletal muscle tissue were isolated by homogenisation, various centrifugation steps and separation on a glycerol density gradient (5-40%) (Methods as described in [19;81]. The glycogen particles (assayed by enzymatic glucose determination after acid hydrolysis) were mainly recovered in the middle fractions, whereas the GLUT4 protein (assayed by Western blot, as in paper III) was mainly recovered in the pellet, where a membrane-incorporated protein is expected to be found (figure 5).

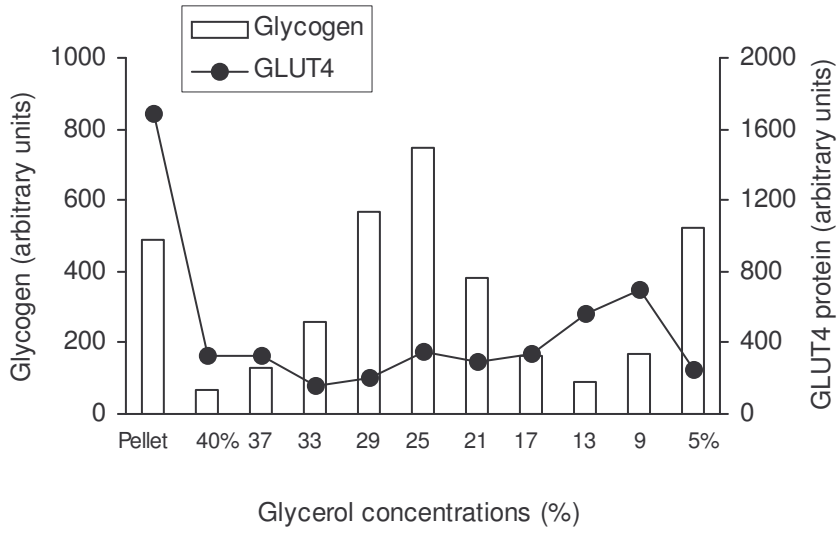

Figure 5: Recovery of glycogen and GLUT4 protein in density gradients. One ml of partly purified glycogen particles ('crude particles') were loaded on a 5-step glycerol gradient (3 ml of 5%, and 2 ml of

10%, 20%, 30% and 40% glycerol in Tris-EDTA buffer) and centrifuged for 2h at 100,000g (4°C). The pellet (resuspended in Tris-EDTA buffer) and ten 1 ml fractions were harvested and determined for glycogen (by enzymatic assesment after acid hydrolysis) and GLUT4 protein content (Western blotting).

- Enzymatic disruption of the glycogen particles with α-amylase prior to the glycerol density gradient centrifugation did not cause a different localisation of GLUT4 protein in the glycerol fraction compared to separation without prior enzymatic treatment.
- After GLUT4 immunoprecipitation of a muscle homogenate, neither glycogen, nor glycogen synthase or phosphorylase (enzymes that are structurally bound to glycogen particles [82]) could be detected in the immunopellet.
- After immunoprecipitation of a muscle homogenate with various antibodies (Gm, R(5)1 and R(5)2; kindly donated by Bo Falck Hansen, Novo Nordisk, Bagsvaerd, DK) against subunits of protein phosphatases, structurally bound to glycogen particles, no GLUT4 protein could be detected on Western blots of immunopellets.
- Rat hindlimbs were loaded with tritiated glycogen by perfusing them post-contractions with 20.000 μU/ml insulin and ^{3}H-glucose for 2 h. After subsequent muscle homogenate immunoprecipitation for GLUT4, the immunopellet did not contain any radioactivity.
- Rat hindlimbs were perfused and fixed consecutively with procaine hydrochloride and paraformaldehyde, and soleus and EDL (extensor digitorum longus) muscles were excised (for detailed method description, see [28;214]). Teased single fibres (30 per well) were incubated with primary antibodies against GLUT4 (mouse; F-27) and glycogen synthase (rabbit; kindly donated by Oluf Pedersen, Steno Diabetes Centre, Copenhagen) and subsequently with fluorescein-conjugated (glycogen synthase: green) and Texas red-conjugated (GLUT4: red) secondary antibodies. The fibres were mounted on glasses and overlap/co-localisation of both proteins (green + red = yellow) was evaluated by superimposition with a fluorescence confocal microscope [214]. Glycogen synthase (like glycogen) was found at many different locations throughout the fibre, as reported in the literature [90;181], whereas GLUT4 was mainly located in the vicinity of nuclei. Co-localisation of both proteins did occur occasionally, but the low resolution did not allow any causal interpretation (see figure 6).

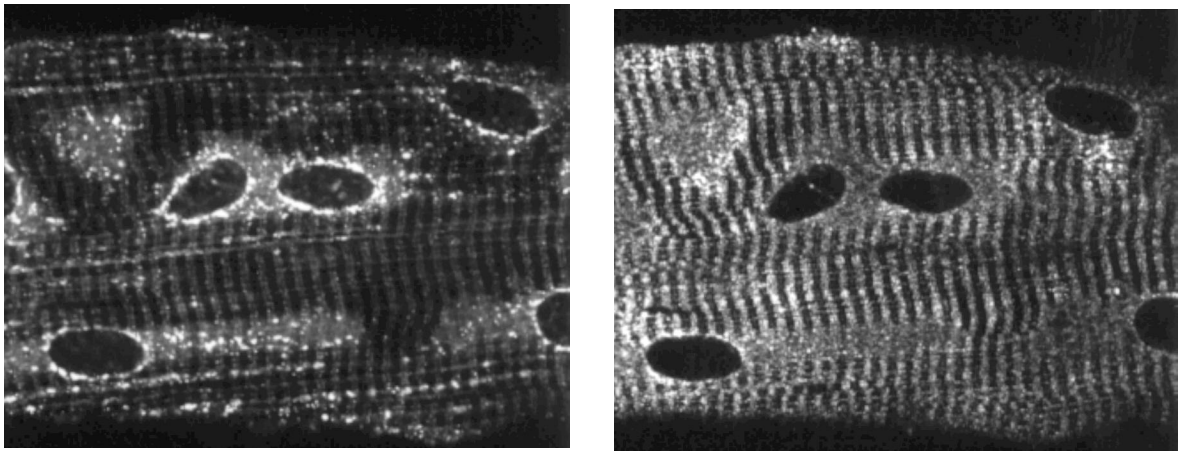

Figure 6: Localisation of GLUT4 (left panel) and glycogen synthase (GS; right panel) in a region of a glycogen-depleted soleus muscle fibre.

In conclusion, the above mentioned results do not support the hypothesis that a strong binding (which persists during agressive isolation methods like immunoprecipitation and multiple centrifugation steps) between glycogen particles and GLUT4-containing vesicles exists in skeletal muscle. This does, however, not exclude that a weaker affinity may exist in muscle cells *in vivo*. The results from the colocalisation study indicate that –due to the low microscopic resolution and the abundant presence of glycogen synthase- further histological examination with electron microscopy needs to be performed in order to exclude or accept a possible GLUT4/glycogen interaction in skeletal muscle.

How is glycogen synthase activated during muscle contractions?

In order to fuel the glycolysis pathway with G6P, contracting skeletal muscle exhibits a large net breakdown of glycogen. Still, it has been shown in rodents and humans that also glycogen synthesis and activation of glycogen synthase (GS) occurs in active muscle at work [10;31;142;172;228], although the effect drowns in the larger rate of glycogenolysis. It has long been established that muscle glycogen undergoes constant turnover, alternately incorporating and releasing glucose units [254].

It is presently unclear why these two energy-consuming processes, glycogenesis and glycogenolysis, also occur simultaneously in opposite directions in a situation (exercise) where every ATP molecule is so precious. However, the exercise activation of GS may prove advantageous for the immediate start and the insulin-independence of glycogen repletion in

recovery from exercise [93]. The signalling pathway for exercise activation of GS is not known, but it is different from the way insulin stimulates GS (for review see [112]). Because GS is bound on glycogen particles and may change its activity or its susceptibility to activation when glycogen disappears in the muscle cells and GS becomes free in solution, we have hypothesised that the glycogen content in the muscle is involved in the regulation of GS activity.

In perfused hindlimb experiments, we have measured GS activity (fractional velocity and I-form)[9] in rested and contracted muscle samples with varying glycogen content (Method as described in Paper III).

The results (figure 7) indicate that the GS is hardly activated by contractions when pre-contraction glycogen levels are high. When calculating correlations for the data of rested and contracted muscles combined, GS fractional velocity (r=-0.86; $P<0.001$) and I-form (r=-0.69; $P<0.05$) are significantly negatively linearly correlated with muscle glycogen content. Although previous studies have shown that GS activity is negatively correlated with the muscle glycogen content [67;300], we now suggest that GS activation during exercise is mainly the result of the decrease in muscle glycogen content. Furthermore, as judged from the GS I-form data (figure 7, right panel), it looks as if there exists a critical glycogen concentration above which GS does not get activated by contractions. In conclusion, activation of GS in exercising muscle could be due to a decrease in glycogen content.

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[9] *In vitro* glycogen synthase activity is conventionally measured in the absence and in the presence of submaximal (0.17 mM) and saturating (3.6 mM; total GS activity) concentrations of the allosteric activator glucose-6-P (G6P). Ratio's of activities measured at 0 to 3.6, and 0.17 to 3.6 mM G6P are referred to as the I-form (G6P-independent form) and fractional velocity, respectively.

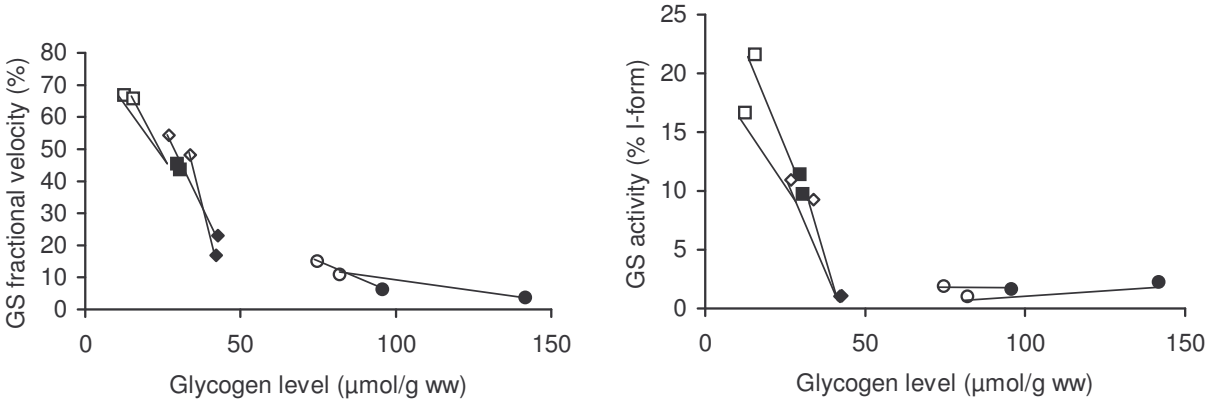

Figure 7: GS activity, expressed as fractional velocity (left panel) or I-form (right panel), in muscle samples with high (circles), normal (diamonds) or low (squares) glycogen levels, measured before (filled symbols) and after (open symbols) electrically stimulated contractions. Each symbol is the mean of 4-9 determinations of white or red gastrocnemius muscle tissue. Pre- and post-contraction GS activities are linked with a solid line.

With our experiments, we have shown that GS activation during exercise only occurs when muscle glycogen levels are low. It remains obscure why there exists a mechanism which upregulates glycogen synthesis in glycogen-depleted, working muscles. One would expect the opposite, that in situations of increased glucose needs (contractions) and decreased intracellular glucose availability (glycogen depletion), all the entering glucose is directed towards glycolysis. However, we may deal here with a protective effect, presumably because complete glycogen depletion is too hazardous for the muscle cells and is therefore protected by activation of GS under these conditions.

How does glycogen inhibit insulin-stimulated glycogen synthesis?

As discussed in Chapter 3 (*Increased post-exercise insulin action on glycogen synthase*), numerous studies have provided evidence for glycogen, as end-product of glycogen synthesis, playing a regulatory role (negative feedback) in insulin activation of glycogen synthase (GS). However, the mechanism of such a controlling mechanism remains unknown. Some of our recent and preliminary findings may provide a mechanistic basis for glycogen regulation of insulin-activated glycogen synthase.

Rats (~100g) were preconditioned by a combination of swimming exercise (see Methods section in Paper V) and diet to obtain highly different muscle glycogen levels (fourfold difference between HG, high glycogen and LG, low glycogen). Rat hindlimbs were exposed during isolated perfusion

for 25 min to 0, 100 or 10000 µU/ml insulin, red gastrocnemius muscles (mixed fibre type composition) were excised. GS activity was determined with an *in vitro* activity assay [258] and muscle GS protein content was assessed with conventional Western blot techniques (GS antibody kindly donated by Oluf Pedersen).

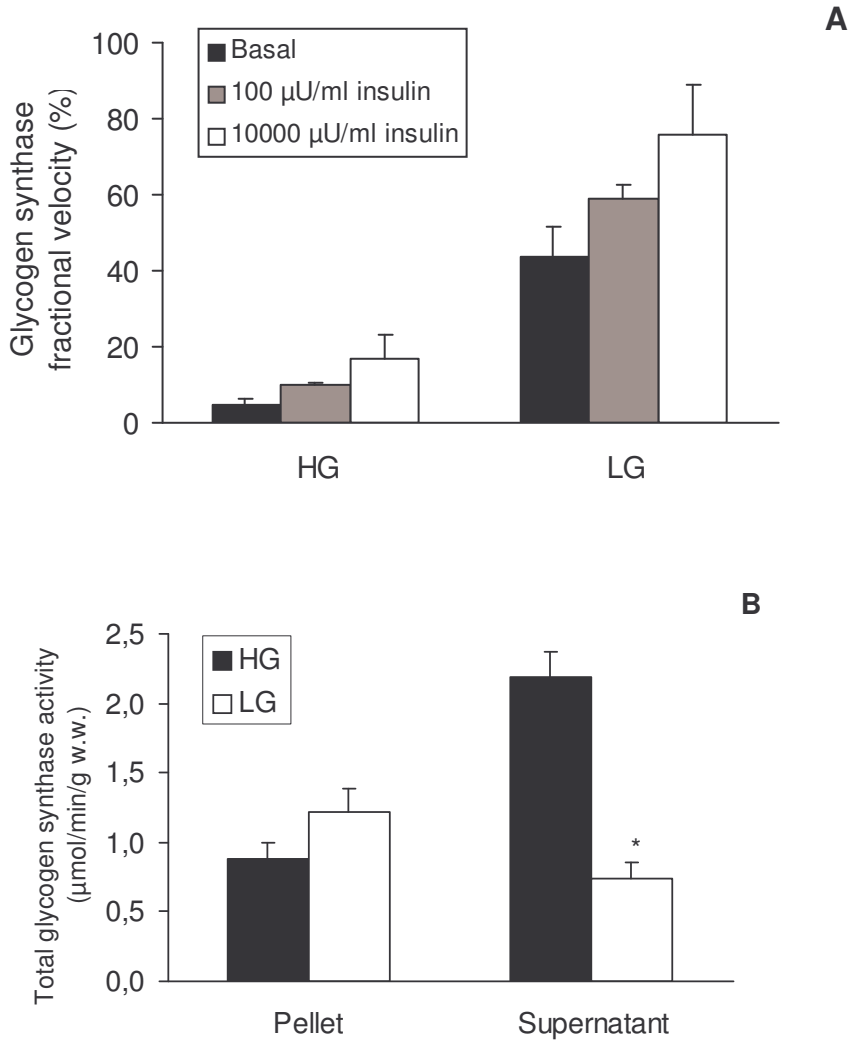

Figure 8: *A:* Insulin stimulated GS activity (fractional velocity) in homogenates of muscles with high (HG) and low (LG) glycogen content (red gastrocnemius, N=5-9). *B*: Total GS activity in pellet and supernatant of a 2700 g spin of muscle homogenate (red gastrocnemius, N=8, mean±SEM). * Significant difference compared with HG

The results show that GS fractional velocity (figure 8A) and I-form (data not shown) were markedly higher in basal glycogen-depleted (LG) muscles than in glycogen supercompensated muscles (HG). Insulin increased GS activity in both HG and LG muscles in a dose-dependent

manner, although the increase was more pronounced in the latter group. Thus, glycogen seems a much more powerful regulator of GS activity than insulin.

Subsequently, we have hypothesised that this different activation pattern of GS by insulin may be due to a different localisation of GS in muscles with high and low glycogen content. We have measured GS activity and GS protein content in the pellet and supernatant of a crude muscle homogenate centrifugation (2700 *g*). Surprisingly, as judged by the total GS activity (figure 8B) and Western blots (data not shown), the majority of the GS protein in HG muscles was found in the supernatant fraction, whereas in LG muscles, the GS protein was predominantly recovered in the pellet. GS is known to be tightly bound to glycogen granules [19;206]. Therefore, it could be hypothesised that in the absence of glycogen particles (e.g. glycogen depletion) muscle GS is bound to other structures, which we recover in the pellet. Because GS is found in the pellet of a low-speed centrifugation, possible interaction of GS with heave cell organelles, such as nuclei, was hypothesised. The absence of GS protein (determined by Western blotting) in an isolated nuclei fraction from glycogen-depleted skeletal muscle tissue suggests that GS is not translocated to the nuclei under glycogen depleted conditions (Kristiansen, Derave & Richter, unpublished observations). Microscopic immuno-localisation of GS was found to be more linearly patterned in LG (figure 9, left panel), compared to HG (figure 9, right panel) muscle cells (Ploug, Derave, Langfort, Ralston & Richter; unpublished observations).

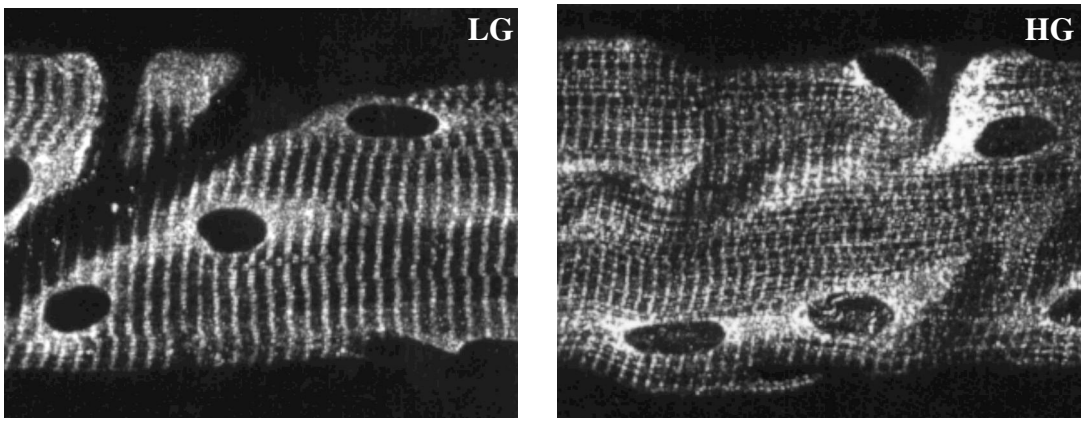

Figure 9: Visualisation of the distribution of glycogen synthase (GS) in a single muscle fibre of an extensor digitorum longus (EDL) muscle of rats with low (left panel) or high (right panel) muscle glycogen content. Note the multiple nuclei (circular structures) throughout the fibre and the capillary (dark band) overlying the fibre.

With this microscopic resolution (~1000x), it is not possible to visually investigate the interaction with cell organelles or filamentous structures. Recent studies in cultured cells, however,

have reported translocation of GS and binding of one of its subunits with actin filaments [17;88]. Further experiments with biochemical methods are in progress (Nielsen, Derave, Kristiansen & Richter). Differences in catalytic properties of GS when free in solution or bound to glycogen particles have long been hypothesised and could suggest a link between the differences in localisation and activity of GS in HG and LG muscles, as seen in the present experiments. Interestingly, this hypothesis is strengthened by our observation that the %I-form and fractional velocity of the GS recovered in the pellet is somewhat, yet significantly higher than in the supernatant (data not shown).

There exists evidence that PKB plays an important role as an intermediate in the insulin signal leading to GS activation, by inactivating glycogen synthase kinase-3 (GSK3), an enzyme that inactivates GS [63;194;263;264], reviewed in [112]. In Paper V, we have demonstrated that PKB activation in response to insulin stimulation is inversely related to the muscle glycogen content. Thus, inhibition of insulin activation of GS in HG muscles, as shown in figure 8A, may in part result from a downregulation of the insulin signal at the level of PKB.

In conclusion, exercise results in increased muscle sensitivity to insulin activation of GS, a process which may in part be explained by the decrease in muscle glycogen concentrations. Glycogen-dependent differences in intracellular localisation of GS and interaction with the downstream insulin signalling cascade are proposed as possible mechanisms.

CHAPTER 5: GENERAL DISCUSSION

The acquisition of new knowledge
generates new and more challenging questions
(Basic premise of science)

Major findings:

In this chapter, I will discuss the significance of the results described in the previous chapter. It is essential to read through the 5 papers in the appendix before turning to this chapter. Below, I have summarized the major new findings.

1. Hypoxia, in contrast to contractions, stimulates muscle glucose uptake independently of A_1-adenosine receptor stimulation (Paper I).
2. Contractions and hypoxia stimulate muscle glucose uptake additively when applied in a submaximal dose (Paper I), but not additively when applied in a maximal dose (Paper II).
3. Protein kinase C is involved in signalling of contraction-, but not hypoxia-stimulation of muscle glucose transport (Paper II).
4. Persistent increased glucose transport rate in muscle after exercise, when deprived from carbohydrate ingestion, is due to persistent GLUT4 transporters at the muscle surface membrane (Paper III).
5. Glucose transport and cell surface GLUT4 content in contracting fast-twitch, but not slow-twitch muscle are dependent on the muscle glycogen content (Paper III).
6. Contraction activation of AMPK is dependent on the muscle glycogen content in an inverse manner (Paper IV)
7. In slow-twitch muscle, inhibition of contraction activation of AMPK does not affect glucose transport, indicating, in contrast to the prevailing opinion, that AMPK is not an essential contraction signalling intermediate for glucose transport in this muscle type (Paper IV).
8. Activation of muscle glucose transport and cell surface GLUT4 content in response to a submaximal or maximal insulin stimulus is dependent on the glycogen content in fast-twitch, but not slow-twitch muscle (Paper V).

9. Insulin activation of protein kinase B (PKB), but not further upstream insulin signalling intermediates, is dependent on muscle glycogen content and may provide an important process in regulation of muscle insulin action (Paper V).

Contractions and hypoxia are different stimuli of muscle glucose transport

The mechanisms by which contractions and hypoxia stimulate muscle glucose transport are believed to be identical, after the observation that the effects of both stimuli on glucose transport are not additive [46]. Based on this dogma, hypoxia seems accepted as a model to study contraction stimulation of glucose transport in incubated muscles. However, because the evidence whereupon this assumption is based, is very limited, we have decided to critically investigate the mechanisms by which hypoxia and contractions stimulate muscle glucose transport. Adenosine [266] and protein kinase C (PKC) [144;145;226] have been proposed to be involved in the contraction stimulation of muscle glucose transport. Therefore, we performed two studies to investigate the involvement of adenosine (Paper I) and PKC (Paper II) in the mediation of hypoxia and contraction effects and we have re-evaluated their kinetics and additivity in the stimulation of muscle glucose uptake/transport.

Firstly, the results from Paper I in the perfused rat hindlimb show that adenosine receptor antagonism by 8-cyclopentyl-1,3-dipropyl-xanthine (CPDPX, [38]) does not affect hypoxia-stimulated glucose uptake in the presence nor absence of submaximal insulin concentrations. In contrast, we confirm a reduction of contraction-induced glucose uptake by 25% by CPDPX in the presence of insulin (Paper I). The notion that adenosine is produced in muscle and accumulated in the interstitium around the muscle cells both during hypoxia [193] as during contractions [126], suggests a differential role of adenosine in regulating glucose uptake in hypoxic and contracting muscles. Secondly, after 60 min of hypoxia (0% oxygen hindlimb perfusion) muscle glucose uptake did not further increase during an additional 30 min of hypoxia, whereas it increased by an additional 60% when applying electrically stimulated contractions (Paper I). This signifies that the contraction stimulus is not simply a result of decreased intracellular oxygen tension and that hypoxia and contractions are complementary, rather than identical in the stimulation of muscle glucose transport. Thirdly, the maximal effect of contractions on muscle glucose uptake and transport is twice as high as the maximal effect of hypoxia, mainly in slow-twitch fibres (Paper II). Fourthly, the time-course of glucose transport activation by hypoxia and contractions is markedly different (Paper I & II). Fifthly, synergistic activation of glucose uptake is observed when muscles are exposed to insulin and contractions but not when exposed to insulin and hypoxia (Paper I).

Finally, two different microbial products, calphostin C and wortmannin, the former a specific [171] and the latter an unspecific [202] inhibitor of conventional PKCs, partly or completely inhibit contraction-stimulated glucose transport, indicating that cPKCs are involved in contraction signalling leading to glucose transport (Paper II). The finding that hypoxia-stimulated glucose transport is insensitive to these drugs strongly suggests different signalling mechanisms for hypoxia and contractions (Paper II).

In conclusion, we have presented accumulating evidence severely challenging the general opinion that hypoxia and contractions are identical stimuli for muscle glucose transport. Therefore, hypoxia stimulation of glucose transport should not be taken as representative for contraction stimulation. In addition, we provide further evidence that adenosine and PKC are involved in the initiation or mediation of the contraction signal leading to increased muscle glucose transport.

Glycogen regulates contraction stimulation of glucose transport and glycogen synthase

The intracellular glucose availability seems to regulate the entrance of blood-borne glucose into muscle cells, as judged by the negative correlation between muscle glycogen content and contraction-induced glucose uptake, observed a decade ago by my supervisors Peter Hespel & Erik A. Richter [131]. They have shown that in the perfused rat hindlimb, the contraction-induced glucose uptake rate was two-fold higher in glycogen-depleted compared to glycogen supercompensated muscles [131]. Approximately 25% of the difference in glucose uptake could be explained by differences in glucose transport rate, as judged by the uptake of radiolabelled 3-O-methylglucose. This left them with the assumption that also other processes, like inhibition of hexokinase by accumulated G6P concentrations in high glycogen muscles, account for an important part of the effect. However, the recent observation by Wojtaszewski, Jakobsen & Richter that the use of the non-metabolisable 3-O-methylglucose can lead to serious underestimation of the actual glucose transport rate and that 2-deoxy-glucose should be used instead [291], prompted us to re-evaluate the classical Hespel & Richter study. In Paper III we now report that contraction-induced glucose transport, rated by the uptake of 2-deoxy-glucose, is indeed 2-fold higher in glycogen-depleted compared to glycogen-supercompensated muscles in fast-twitch type and mixed type muscles (representing 95% of the total rat musculature). This would imply that a major part of the regulatory effect of glycogen on glucose uptake involves the control of GLUT4 mobilisation, rather than the control of glycolytic enzymes. To directly test this, we have measured the number of active

GLUT4 transporters at the cell surface membrane by means of a surface labelling technique with bis-mannose derivatives [189]. The results (Paper III) show that the contraction-induced increase in cell surface GLUT4 content in the fast-twitch plantaris muscle was twice as high ($P<0.05$) in the glycogen-depleted compared to the glycogen-supercompensated state. This strongly confirms our novel conception that contraction induction of GLUT4 mobilisation is controlled by intracellular glycogen availability.

The next step was to investigate the mechanism underlying this regulatory process. The fact that glycogen is a polysaccharide (polymer of glucose units) and that it is generally believed that GLUT4 mobilisation is under regulation of proteins, such as signalling cascades of protein kinases, makes it difficult to understand their regulatory link. Still, glycogen particles are known to bind enzymes involved in glycogen metabolism [198;277] and are actually cell organelles on themselves, called glycosomes [239]. By analogy, glycogen particles could bind GLUT4 proteins and thereby control their activation, a hypothesis which has frequently been put forward [123;148]. We have studied this possibility (See Chapter 4: *Are GLUT4-containing vesicles structurally linked to glycogen particles?*), but the results from the different methodological strategies equivocally lead to the conclusion that GLUT4 is not bound or co-located with glycogen particles, suggesting that the above-mentioned glycogen/GLUT4 hypothesis is false.

This lead us to the second possibility, that glycogen regulates GLUT4 translocation through an indirect process in contracting muscles, possibly by interacting on the contraction signalling cascade. Although the contraction signalling cascade is largely unknown, 5'AMP-activated protein kinase (AMPK) has recently been suggested to be involved [66;121;197]. AMPK is a wide-spread fuel gauge of the mammalian cell, linking situations of increased intracellular energy demand (e.g. during contractions) with increased energy supply, such as increased fatty acid mobilisation and possibly also glucose transport in contracting skeletal muscle cells [116;117;216;285]. In Paper IV, we show that AMPK activation during contractions is closely related to the glycogen content of the muscle cells. Thus, in contracting fast-twitch fibres, high glycogen levels inhibit AMPK activation and – possibly causally linked to that – glucose transport rate, whereas low glycogen levels exert the opposite effect on both processes (Paper IV). Therefore, AMPK is an accurate sensor of intracellular glucose availability (glycogen content), and is maybe involved in the mediation of a signal leading to increased glucose transport in contracting fast-twitch skeletal muscle cells.

In conclusion, contraction-induced glucose transport and GLUT4 translocation in fast-twitch skeletal muscle are dependent on muscle glycogen levels. This is probably due to an interaction of glycogen with the contraction signalling cascade, rather than a direct physical interaction with GLUT4 vesicles.

Glycogen-dependent insulin action on glucose transport and glycogen synthase

It has been shown that the insulin-stimulated glucose transport rate is negatively correlated with the muscle glycogen content [151]. It has been proposed that the mechanism of increased insulin sensitivity of glucose transport following exercise [227], is related to the muscle glycogen depletion, inducing increased insulin action. We have investigated the effect of varying muscle glycogen levels on insulin-stimulated glucose transport, GLUT4 mobilisation and insulin signalling (Paper V). In perfused fast-twitch muscles, but not in slow-twitch muscles, there is a marked effect of glycogen content on both insulin sensitivity and responsiveness of glucose transport. In additional experiments on incubated epitrochlearis muscles, cell surface GLUT4 content was evaluated with the aid of the biotinylated bis-mannose photolabel [175]. The surface GLUT4 content was significantly higher (2- to 3-fold) in glycogen-depleted compared to glycogen supercompensated muscles, both at submaximal and at maximal insulin concentrations. Initial insulin signalling events, as evaluated by insulin receptor tyrosine kinase activity (IRTK) and IRS1-associated PI3K activity, are not affected by glycogen levels. Interestingly, however, a more downstream event, protein kinase B (PKB) activation, is glycogen-dependent. This would indicate that glycogen depletion facilitates, and glycogen supercompensation inhibits insulin-induced PKB activation by a PI3K-independent pathway. It is surprising to see that insulin-stimulated glucose transport can vary to a large extent without variation in PI3K activity. Interestingly, four papers, published this year, have reported situations where altered insulin sensitivity correlates either with PKB activity or with PI3K activity, but never with both. Two of them showed that hyperglycaemia-induced insulin resistance involves downregulation of PKB, but not PI3K [178;251]. Oppositely, others have found insulin resistance to be accompanied by decreased PI3K, but not PKB activity [164;165]. Thus, recent evidence suggests that PKB is not only subject to PI3K-dependent phosphorylation, but is also influenced by cross-talk of other signals. The present study (Paper V) suggests that this other signal is sensitive to the muscle glycogen content.

As shown in Chapter 4 (*How does glycogen inhibit insulin-stimulated glycogen synthesis?*) also insulin-stimulated glycogen synthase (GS) activation is dependent on muscle glycogen. In fact, the

effect of insulin on GS activity seems of secondary importance, compared with the large effect of muscle glycogen content. In the search for the mechanism of glycogen-dependent GS activation, we propose that differences in GS localisation as well as in insulin signalling are involved. Thus, two of the important and rate-controlling steps in glycogen synthesis, *i.e.* glucose transport and glycogen synthase activation, are feedback-inhibited by the final product, glycogen. This mechanism assures that glycogen is not needlessly formed in muscle when glycogen is abundant and may even protect the cell from overaccumulation, as muscle cells seem to have a well-defined upper limit of glycogen storage capacity [114]. However, the evidence presented in this thesis suggests that feedback-inhibition by glycogen does not -or not only- occur through the classical 'allosteric' product inhibition, but rather –or also- through a negative 'feedback loop', involving signalling pathway intermediates. In conclusion, muscle glycogen-dependent activation of PKB in response to insulin may provide an explanation for the effect of glycogen depletion/exercise on insulin-stimulated glucose transport, GLUT4 translocation and GS activation.

The missing link between contractions and insulin action: a hypothesis

Insulin stimulation of muscle glucose transport is positively affected by exercise in two ways [101]. Firstly, the simultaneous effect of insulin and exercise on glucose transport is higher than would be expected from the sum of the effects of either stimulus alone [70]. Thus, exercise and insulin act synergistically on glucose uptake or in other words, exercise enhances concurrent insulin action. Secondly, in the hours following an exercise bout, muscle insulin sensitivity is persistently increased. The mechanism underlying these effects of exercise on simultaneous and subsequent insulin action is not known, but it may be a common one. An overview of possible common modulators:

A_1-adenosine receptor antagonism by 8-cyclopentyl-1,3-dipropyl-xanthine (CPDPX) counteracts the synergistic action of insulin and contractions on muscle glucose uptake ([266], Paper I). Additionally, Jozef Langfort and colleagues have reported that adenosine partly accounts for the increased insulin sensitivity in the period following exercise [182]. Thus, adenosine may play a role in both processes.

Glycogen depletion, a major feature during muscle contractions, enhances insulin stimulation of glucose transport and PKB activity (Paper V). In addition, insulin activation of human muscle glucose uptake and PKB activity is enhanced immediately following glycogen-depleting exercise

[259]. These data indicate that PKB is possibly an important signalling intermediate, integrating exercise and insulin signals; exercise activating PKB by a glycogen-dependent mechanism, and insulin activating PKB by a PI3K-dependent mechanism (Figure 10).

Thus, both adenosine and glycogen/PKB are now assumed to be involved in the interaction of exercise and insulin action in muscle. It is tempting to hypothesise that both factors are in fact part of the same process or signalling pathway.

A first possibility is that PKB is not only activated by phosphorylation by phosphoinositide dependent kinases (PDKs) but also by unknown contraction-dependent kinases. Recently, PKB has been shown to be activatable by adenosine 3',5'-cyclic monophosphate (cAMP) in a PI3K-independent manner [240]. This probably involves cAMP dependent protein kinases (PKAs) [71;89]. Interestingly, the main mode of action of adenosine receptors is by altering [decreasing (A_1-receptors) or increasing (A_2-receptors)] cytoplasmic cAMP levels [219] and also adrenergic receptors induce their action through cAMP [49;210;265]. An increase in the second messenger cAMP concentration has been shown to reduce insulin sensitivity in muscle [213]. Therefore, it is possible that the synergistic stimulation of glucose uptake by insulin and exercise is a result of the enhancing effect of contractions on PKB activity, mediated by stimulated A_1-adenosine receptors and a resulting decrease in cAMP (figure 10). How glycogen is involved in this system is not exactly clear. To my knowledge, it has not directly been shown that glycogen depletion in itself can enhance adenosine release in skeletal muscle. However, glycogen depletion surely impairs ATP regeneration in working muscle and will therefore lead to AMP accumulation [32;105], which will probably favour adenosine release [125]. Therefore, it remains to be established whether the effect of glycogen depletion on PKB activity also acts through the adenosine-mediated exercise effect on PKB.

A second possibility for explaining the enhanced insulin activation of PKB by exercise/glycogen depletion could be that glycogen continuously exerts a direct inhibiting effect on PKB activity, *e.g.* by binding PKB or one of its activators. Exercise would consequently relieve this inhibition by depleting glycogen stores. Adenosine would then regulate exercise effects on insulin action by a different mechanism than glycogen/PKB.

In conclusion, exercise, prior to or during insulin stimulation, enhances insulin action on glucose transport in skeletal muscle. Exercise probably modulates downstream insulin signalling at the level of PKB, possibly through an adenosine/cAMP-mediated signalling pathway, which is enhanced by glycogen depletion.

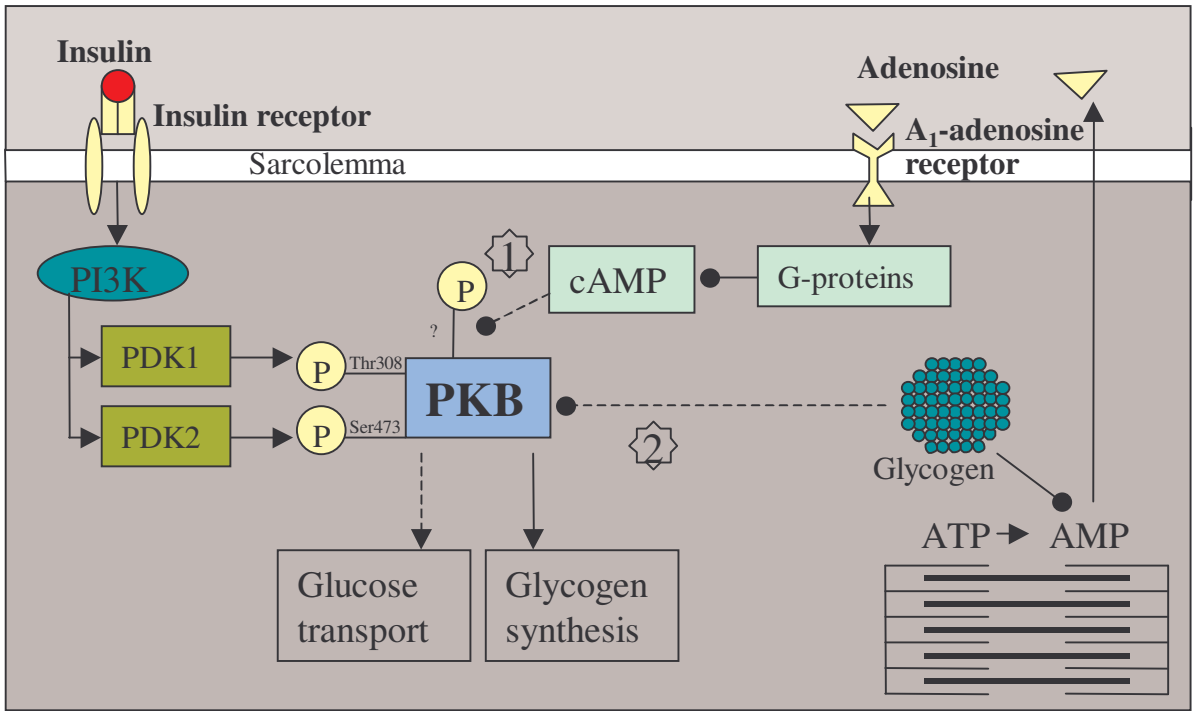

Figure 10: Hypothesised model of protein kinase B (PKB) as an integrative protein of interacting exercise and insulin signals leading to increased glucose transport and glycogen synthesis in a skeletal muscle cell. *First hypothesis*: full activation of PKB requires phosphorylation at distinct serine and threonine residues by both PI3K-dependent (insulin: phosphoinositide-dependent kinases, PDKs) and –independent (exercise: cAMP dependent protein kinases, PKAs) kinases. *Second hypothesis:* exercise-induced glycogen depletion relieves the constant inhibition of glycogen on PDK-mediated PKB phosphorylation.

The isoforms of adenosine receptors and their distribution are largely undiscovered in skeletal muscle, as is their signal transduction pathway [132]. Moreover, there is severe controversy in the literature concerning the effect of adenosine on skeletal muscle insulin sensitivity, which has been shown to be increased [62;111], unchanged ([266], Paper I) or decreased [39;48]. Therefore, the characterisation of adenosine action in skeletal muscle cells and its involvement in exercise metabolism awaits intensive further research and clarification.

The slow-twitch fibre type: a troublesome child?

Despite numerous experiments and trials, we have never observed an effect of varying muscle glycogen levels on the regulation of glucose utilisation in the soleus muscle, a muscle without fast-twitch glycolytic fibres [7]. Oppositely, in the white region of the gastrocnemius, containing 100% fast-twitch fibres, an effect of muscle glycogen content on glucose transport was always observed, without a single exception. This prompts us to believe that the role of glycogen on glucose utilisation is fibre-type specific. It could be argued that a regulatory role for glycogen in slow-twitch fibres is active, but too weak to cause an observable effect (quantitative differences between fibre types), or simply is absent (qualitative differences). In the rat, glycogen concentration is lower ([245], Paper III) and the GLUT4 expression higher [196] in slow-twitch compared to fast-twitch muscles. Therefore, the lack of effect of glycogen on contracting soleus glucose transport could be attributed to the low absolute glycogen concentration and the high GLUT4 protein concentration, making a regulatory role of the former on the latter less favourable (i.e. a quantitative difference). However, regarding the possible role of AMPK in contraction signalling of muscle glucose transport (Paper IV), the results from fast- and slow-twitch fibres are clearly opposite (i.e. qualitative difference). In fast-twitch fibres, AMPK activity and glucose transport rate are significantly positively correlated, supporting a role for AMPK in contraction signalling. In slow-twitch fibres, however, glucose transport is insensitive to inhibition of AMPK activity, indicating that AMPK is not essential for contraction signalling to glucose transport in this fibre type. Also regarding the involvement of PKC in contraction signalling to glucose transport, fibre type differences exist. Calphostin C inhibited contraction-induced glucose transport by 60% ($P<0.05$) in the fast-twitch white gastrocnemius, but not at all in the slow-twitch soleus (Paper II). Finally, the role of adenosine in regulating glucose transport is reported to be restricted to slow-twitch muscles [266]. In short, the available data on glycogen, AMPK, PKC and adenosine involvement in contraction-induced glucose transport all suggest different signalling pathways in the different muscle fibre types.

It has been known for a long time that the different fibre types have highly different metabolic characteristics [84;243], but the differences are thought to be more quantitative (e.g. varying concentrations of oxidative enzymes and myoglobin). The present data, however, may suggest that the fibre types have different signalling pathways, with different proteins involved, in stimulating glucose transport during contractions. This provocative view is hard to believe for many exercise physiologists, including ourselves. Maybe we should abandon previous ideas, that the metabolic

regulation in the different fibre types is only quantitatively different. It should be noted that the different fibre types are 'officially' different cell types and express different structural and contractile proteins (e.g. myosin heavy chain proteins [3]). So why not different signalling proteins?

Final conclusions and future directions

In this thesis we provide the first evidence that hypoxia and contractions are different stimuli for muscle glucose transport and probably exhibit different signalling pathways, a finding in sharp contrast with the general opinion. Secondly, we have characterised glycogen as an active molecule in metabolic regulation in fast-twitch, but not slow-twitch skeletal muscle cells. This is based on the novel finding that glycogen operates upon signalling pathways leading to increased GLUT4 mobilisation and glycogen synthase activity in response to exercise and insulin. Finally, and again in contrast with the general opinion, we provide convincing evidence that –at least in slow-twitch skeletal muscle- AMP-activated protein kinase (AMPK) is not an essential signalling intermediate in contraction-induced glucose transport.

A lot of questions about the effects of exercise on muscle glucose transport and insulin action remain unsolved. Although it becomes evident that contractions elicit an intracellular signalling pathway with different starting points and although some of the players of the pathway have been identified, the present knowledge is insufficient to understand the link to the end process, the actual increased glucose transport. The present studies emphasise the importance of the different metabolic characteristics of the different muscle fibre types and the complication that derives from that. AMPK was first studied for its role in the regulation of fatty acid oxidation in contracting muscle and is now thought to be involved in glucose uptake regulation. The same counts for adenosine and nitric oxide (NO), initially described as contraction-induced vasodilators. The transcription and translation rates of many muscle enzymes are upregulated after exercise. Hemodynamics, protein expression and metabolism are clearly interactively regulated processes in the working muscle. Thus, researchers with different interests are apparently all looking for the same contraction signal web with many endpoint effects. The importance of collaboration in the attack of this integrated problem is therefore paramount!

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SAMENVATTING

Dit doctoraatproefschrift behandelt de effecten en het signaalmechanisme van fysieke inspanning/spiercontracties op de glucose-opname en insulinewerking in skeletspieren.

Tijdens fysieke inspanning worden de spieren geconfronteerd met een sterk verhoogde nood aan suikers, voornamelijk onder de vorm van glucose, als brandstof voor de contractiearbeid. Daarom veroorzaken contracties een stijging in de opname van glucose uit de bloedbaan, evenals een afbraak van glycogeen. Een eerste hypothese van dit proefschrift is dat de hoeveelheid glycogeen in de spiercellen een regulerend effect heeft op de snelheid van opname van bloedsuiker, gestimuleerd door insuline en spiercontracties.

Glucosetransport doorheen de spiercelmembraan gebeurt via gefaciliteerde diffusie door transporteiwitten, GLUT4 genaamd. Deze worden geactiveerd door translocatie vanuit opslagplaatsen naar de celmembraan. Het mechanisme dat leidt tot GLUT4 translocatie tijdens spiercontracties is nauwelijks gekend. In dit proefschrift wordt de rol van de signaaleiwitten proteïne kinase C (PKC) en AMP-geactiveerd proteïne kinase (AMPK) en van het autocriene/paracriene hormoon adenosine in dit proces onderzocht.

De resultaten uit deze thesis bevestigen dat glycogeen een regulerende rol speelt in het stimulerend effect van insuline en contracties op het glucosetransport in de spier. We tonen aan dat het aantal actieve GLUT4 eiwitten in de celmembraan afhankelijk is van de spierglycogeeninhoud. De regulatie door glycogeen zou eerder te verklaren zijn door een effect op intermediairen van de signaalwegen van de stimuli dan door een direct effect op de GLUT4 eiwitten.

Contractie-activatie van AMPK, vermoedelijk een signaaleiwit in de contractiestimulus van glucosetransport, is negatief gecorreleerd met de spierglycogeeninhoud. In snelle, maar niet in trage spiervezels, is ook het contractiegeïnduceerde glucosetransport negatief gecorreleerd met de spierglycogeeninhoud. Dit wijst erop dat glycogeenafhankelijke AMPK activatie mogelijk een verklaring biedt voor het effect van glycogeen op het glucosetransport in de arbeidende snelle spiervezels. Daarentegen kan AMPK in trage spiervezels geen essentieel signaaleiwit zijn in de contractie stimulus voor glucosetransport.

De signaaltransductieweg van insuline ter stimulatie van glucosetransport gebeurt via verschillende enzymen, waaronder phosphatidylinositol-3-kinase (PI3K) en proteïne kinase B

(PKB). De activatie door insuline van PKB, maar niet van PI3K, is afhankelijk van de spierglycogeeninhoud. Dit kan mogelijk een verklaring zijn voor de glycogeen-afhankelijkheid van insulinegestimuleerde glucose-opname en voor de verhoogde insuline gevoeligheid na fysieke inspanning.

Algemeen wordt aangenomen dat contracties en hypoxie twee identieke stimuli zijn voor glucose-opname, gebaseerd op de observatie dat het maximaal effect van beide stimuli niet additief is. Aangezien deze stelling op een beperkt aantal gegevens berust, was een tweede objectief van dit onderzoek kritisch na te gaan of hypoxia en contracties inderdaad identieke stimuli zijn voor glucose-opname in de skeletspier, door hun respectievelijke signaalwegen te onderzoeken. De resultaten leiden éénduidig tot de conclusie dat hypoxia en contracties in essentie twee verschillende stimuli zijn voor glucose-opname. Antagonisme van adenosine receptoren en PKC leiden tot verminderde glucose-opname tijdens contracties maar niet tijdens hypoxia. Daarenboven zijn submaximale hypoxia en contracties onder meer fysiologische omstandigheden additieve stimuli voor glucose-opname in de spier.

De resultaten in dit doctoraatsproefschrift hebben geleid tot drie belangrijke nieuwe bevindingen. 1) Contracties en hypoxia zijn twee verschillende stimuli van glucose-opname, met vermoedelijk elk hun eigen signaalweg. 2) Glycogeen reguleert de insuline- en contractie-activatie van glucosetransport en GLUT4 translocatie in de spieren, vermoedelijk door in te werken op de signaalwegen van de stimuli. 3) AMPK is waarschijnlijk geen essentieel intermediair in contractiesignalering van glucosetransport.

SUMMARY

This PhD-thesis deals with the effects and the signal mechanisms of exercise/contractions on glucose uptake and insulin action in skeletal muscle.

During physical activity, muscles are confronted with a markedly increased need for sugars, mainly glucose, as fuel for their contractile work. Therefore, contractions cause an increase in uptake of blood-borne glucose and in glycogen breakdown. In this thesis, we hypothesised that the glycogen content of the muscle cells has a regulatory effect on the rate of glucose uptake, stimulated by insulin and contractions.

Glucose transport across the muscle cell membrane is accomplished by facilitated diffusion through transport proteins, named GLUT4. They are activated by translocation from intracellular storage sites to the outer membrane. The mechanism, leading to GLUT4 translocation during contractions is poorly understood. In this thesis, we have examined the role of the signal proteins, AMP-activated protein kinase (AMPK) and protein kinase C (PKC) and of the autocrine/paracrine hormone adenosine in this process.

The results of our experiments confirm that glycogen plays a regulatory role in the stimulating effect of insulin and contractions on muscle glucose transport. We show that the number of active GLUT4 molecules on the surface membrane is dependent on the muscle glycogen content. The regulation by glycogen would be due to an effect on signalling intermediates, rather than to a direct effect on the GLUT4 proteins.

Contraction activation of AMPK, a presumed signalling intermediate in the contraction stimulus of glucose transport, is negatively correlated to the muscle glycogen content. In fast-twitch, but not slow-twitch fibres, also contraction-induced glucose transport is negatively correlated to muscle glycogen content. This indicates that glycogen-dependent activation of AMPK is possibly responsible for the observed glycogen-dependent glucose uptake in contracting fast-twitch muscle cells. Controversially, AMPK does not seem to be an essential signalling intermediate in the contraction stimulus leading to increased glucose transport.

The signalling pathway by which insulin activates muscle glucose transport involves several proteins, including phosphatidylinositol-3-kinase (PI3K) and protein kinase B (PKB). Insulin activation of PKB, but not PI3K, is dependent on the muscle glycogen content. This may provide an

explanation for the glycogen dependence of insulin stimulated glucose transport and GLUT4 translocation and possibly also for the well-known increased insulin sensitivity following exercise.

It is generally believed that contractions and hypoxia are two identical stimuli for glucose uptake, based upon the observation that the maximal effects of both stimuli are not additive. Because this assumption is based on few experimental background, it was our aim to critically re-evaluate and investigate the characteristics and signalling pathways of hypoxia and contractions as stimuli of muscle glucose uptake. Our results have led to the conclusion the two stimuli are clearly different. Antagonism of adenosine receptors and PKC lead to diminished contraction-induced, but not hypoxia-induced glucose uptake. Furthermore, under more physiological conditions, hypoxia and contractions are additive stimuli for muscle glucose uptake.

The results in this thesis have led to three important new findings. 1) Contractions and hypoxia are two different stimuli of muscle glucose uptake, presumably with different signalling pathways. 2) Glycogen regulates insulin and contraction activation of glucose transport and GLUT4 translocation in skeletal muscle, presumably by interacting on intermediates of their respective signalling pathways. 3) AMPK is probably not an essential intermediate in contraction signalling of glucose transport.

APPENDIX

Paper I: Adenosine and muscle glucose uptake

Paper II: Hypoxia and contraction signaling of glucose transport

Paper III: Glycogen and muscle glucose transport

Paper IV: AMPK and muscle glucose transport

Paper V: Glycogen-dependent PKB activation

GLUT4 is a gate
for a special substrate,
A minuscule hole
with a physiological role,
It's a peptide chain
in a lipid domain,
With its helical coil
twitsted through oil,
A transporting entity
with a carrier identity,
It pulls and it tugs
while the substrate it hugs,
Done excitingly quick
like a magical trick,
And the evidence is strong
that the mode is ping-pong.

Inside it rests
until insulin requests,
And when muscles contract
it's in its contract,
to move to the membrane
and get on the strain,
To bring sugar inside
and fuel the cyclist's ride,
It's a model supreme
that the wise heads did dream,
It stands to the test
and so far it's the best