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MATHEMATICAL MODELLING OF INSULIN SIGNALLING: EFFECTS ON GLUCOSE METABOLISM IN SKELETAL MUSCLE

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ABSTRACT

The use of models to understand complex phenomena is indispensable to the scientific community. The advantage of a model is that it simplifies the phenomena under study. However, a model should be only as complex as required, no more, no less. Furthermore, a model should avoid known or unknown confounding variables that might obscure the interpretations of observations. Within biology, models can be set up in many different ways, such as mathematical, graphical or verbal descriptions of the system under study. In physiology, the systems under study can be the entire animal or organs or cell cultures from it. To study some aspects of the regulation of glucose and energy homeostasis, skeletal muscles is a preferable model, as it is the main consumer of post-prandial glucose, and thus, important for maintaining whole body glucose and energy homeostasis. Incubation of skeletal muscle specimens in a suitable solution is a model-system that has been used during the last century. The availability of oxygen for energy transformation has been of major concern. Therefore, the experimental system has been validated several times with different methods, both experimentally and mathematically.

The result from experimental validations indicates that glycogen content is unequally distributed within the incubated muscle specimens, with the core depleted of glycogen. Furthermore, validation done with the mathematical models describing the experimental systems indicates that oxygen diffusion is sufficient if the following assumptions are valid; homogeneous structure and that the critical value of oxygen pressure is above zero throughout the entire muscle. However, if those assumptions are invalid, the observations of some metabolic and/or signalling data might be invalid. In this thesis, those assumption are validated, with the specific aim to derive mathematical models that can be used to further analyse the metabolic data generated.

Set of ordinary differential equation was used to describe the metabolic data derived from incubation of mouse *extensor digitorum longus* skeletal muscles preparations, *paper 1*. The parameters and constants were identified within the mathematical model, which then, was further analysed. The results indicated that the experimental system suffered from anoxia and that glycogen was depleted during the incubation time. An immunohistochemical approach was used to verify the predictions from the mathematical model on glycogen depletion, *paper 2*. A statistical approach was developed herein that made quantitative studies possible and the results verified the prediction from the mathematical model in *paper 1*. Furthermore, a correlation between fibre type distribution and glycogen depletion was observed, indicating that the assumption on homogeneous glucose handling might be too hard. The existence of anoxia within the incubated muscle specimens was revealed. A novel hypothesis regarding deficient insulin diffusion into the centre of the incubated muscle preparation as the cause for quasi-depletion of glycogen was tested, *paper 3*. The hypothesis was falsified; instead increased insulin signalling was observed in the core of the muscle, correlating with fibre types on the single-cell-level.

In conclusion, the studies presented in this thesis provide evidence that muscle preparations are suffering of anoxia after incubation leading to depletion of glycogen. Furthermore, the assumption on homogeneous glucose handling is falsified. Finally, a mathematical model is provided that can be used to estimate the un-measurable glycogen concentrations and estimate the glucose uptake rate in the superficial fibres.

LIST OF PUBLICATIONS

- 1 **Peter Sogaard**, Mikael Harlén, Yun Chau Long, Ferenc Szekeres, Brian R. Barnes, Alexander V. Chibalin and Juleen R. Zierath. Validation of in vitro incubation of extensor digitorum longus muscle from mice. *Journal of Biological Systems*. 2010, in press
- 2 **Peter Sogaard**, Ferenc Szekeres, Maria Holmström, Dennis Larsson, Mikael Harlén, Pablo Garcia-Roves and Alexander V. Chibalin. Effects on fibre type and diffusion distance on mouse skeletal muscle glycogen content in vitro. *Journal of Cellular Biochemistry*. 2009, 107: 1189-1197.
- 3 **Peter Sogaard**, Ferenc Szekeres, Pablo Garcia-Roves, Dennis Larsson, Alexander V. Chibalin and Juleen R. Zierath. Spatial insulin signalling in isolated skeletal muscle preparations. *Journal of Cellular Biochemistry*. 2010, 109: 943-949.

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LIST OF ABBREVIATIONS

AIRC	Activated insulin/insulin receptor complex
ATP	Adenosine triphosphate
CO ₂	Carbon dioxide
ECDF	Empirical cumulative data distribution function
EDL	Extensor digitorum longus
G6P	Glucose-6-phosphate
<i>G6P</i>	Variable describing the concentration of G6P
GLUT4	Glucose transporter 4
<i>GLY</i>	Variable describing the concentration of glycogen
GS	Glycogen synthase
HIF1- α	Hypoxia induced factor 1 α
IGT	Impaired glucose tolerance
IDF	International Diabetes Federation
KHB	Krebs-Henseleit bicarbonate buffer
MODY	Mature onset diabetes in young
N ₂	Nitrogen
O ₂	Oxygen
OCM	One compartment model
ODE	Ordinary differential equation
OGTT	Oral glucose tolerance test
pAkt	Akt phosphorylated on serine 473
PAS	Periodic-acid-Schiff
PBS	Phosphate buffer solution
PBT	PBS containing 0.2% Triton X-100
PDE	Spatial differential equation
pGSK3	Glycogen synthase kinase phosphorylated on serine 21/9
r	Radius
RGB	Red green blue
T2DM	Diabetes mellitus type 2
Zn	Zinc

1 INTRODUCTION

1.1 OXYGEN; A WASTE PRODUCT

The conditions for life to evolve, as we know it today, were probably the actual formation of bio-molecules [1, 2]. A hypothesis has been put forward of a Zink-world [1, 3] as the place where the photo stable DNA-bases formed [2]. These DNA-bases protected more fragile carbon molecules that were spontaneously formed [2, 4]. Glycolysis, the transformation of glucose to pyruvate, is the most ancient known biochemical pathway, that extracts energy from carbohydrates [5]. It is thought to have evolved before life [4] (figure 1), and it does not require oxygen to function. However, the yield of transformed energy is not high in glycolysis compared to the amount of energy gained when oxygen is used as electron donor.

Oxygen and its derivatives are the most powerful oxidisers known [6]. When cyanobacteria started to use water instead of hydrogen sulphide, Zink or other compounds as donors for electrons; the fixation of carbon became more efficient. As a result, the concentration of the waste product, oxygen, increased in the atmosphere [7], and carbon dioxide decreased. The accumulation of oxygen in the atmosphere and water began 2.5 billion years ago [8], this phenomenon is referred to as The Great Oxygenation Event, which started after that the Earth's minerals became saturated with oxygen and the capturing stopped (figure 1).

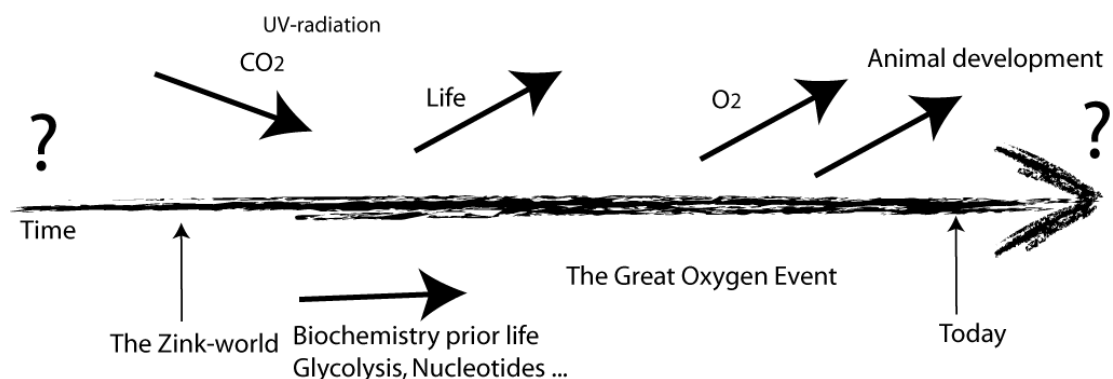


Figure 1. A vast amount of time has passed since the first biochemical components and pathways started to function. There is evidence that a Zink-world was the place that the first biochemical compounds were formed [1, 3]. In a small world protected from UV-radiation, Life had a chance to start [2]. The Great Oxygen Event allowed for more complex animals to be developed.

1.2 ANIMAL EVOLUTION; OXYGEN AND SIZE

The availability of oxygen in water and the atmosphere likely accelerated the evolution of multicellular eukaryote organisms as they started to use aerobic metabolism instead of anaerobic [9] (figure 1). The origin of cells, as prokaryotes, is not known, but they were present on Earth 3.5 billion years ago [6]. The first prokaryotes depended upon anaerobic conditions. The formation of the more complex eukaryote cells took place in the same era as oxygen started to accumulate in the atmosphere and water [10, 11]. According to the endosymbiotic theory [12], a symbiosis between two prokaryotes cells, one relaying upon anaerobic metabolism, the other upon aerobic metabolism,

occurred. The ancient aerobic prokaryote was engulfed by the anaerobic cell; this is hypothesised to be the origin of mitochondria [9].

The evolution of animals' origins in single-celled protozoan's; the unicellular ancestry of animal development [13]. The protozoan's had their weight supported by the surrounding water and were able to move by simple organelles e.g., cilia. The evolution of large and more complex animals necessitated the development of support and locomotion systems. Animals use their muscular and skeletal systems for support, locomotion, and to maintaining their shape. However, in a multicellular organism, diffusion of oxygen is limiting the use of aerobic metabolism. This feature is overcome by the introduction of a circulating system where oxygen is continuously delivered to the single cells. The circulating system also delivers the nutrients needed for the single cell to maintain its energy homeostasis.

Oxygen is available to animals as a gas, which by respiration is first transferred to haemoglobin in the circulating blood, and then further into the skeletal muscle cells. In the muscle cells, oxygen is stored bound to myoglobin until it is further used as an electron donor during oxidative phosphorylation in the mitochondria.

Animal size is hypothesized to be dependent upon the pressure of oxygen in the atmosphere [14], as the size of the respiratory compartment i.e. lungs or gills is crucial, and assumed to be proportional to the overall size of the animal. Oxygen concentration have fluctuated in the environment during the Earth eras [15], sometimes positively correlating with the size of recorded animal fossils. Recently, this hypothesis was questioned [15], arguing that decreased oxygen pressure could be compensated by increasing the capillary density; however, consensus has been reached about the importance of the availability of oxygen for each cell.

1.3 SYSTEMS BIOLOGY

Systems biology is partially a new field where the scientific methods used are from many disparate scientific fields [16, 17]. In the early 2000, Kitano put forward a definition of Systems Biology [18]. Since then, several attempts have been made to modify the definition. The author's scientific field flavours the definition. However, a uniting part in most definitions is that systems biology uses holism as scientific method when attempting to understand the essential mechanisms that control the biological questions under study [19]. This is in contrast to the reductionist approach that is predominantly used by scientist within Life Sciences [19]. The reductionist approach has successfully identified many components and some interactions, but has so far been unsuccessful to explain how system properties emerge [20]. One of the goals for the systems biologist is to discover new emergent properties from data that has been collected over the past years and still are collected today.

1.3.1 Bottom up versus top down

Traditionally, mathematical models of molecular systems are described from a bottom-up view [21], where the molecular interactions predominantly are described by phenomenological functions e.g., Michaelis-Menten kinetics, Hill equations etc. These models are often extensive, with a lot of parameters with unknown numerical values. The qualitative behaviour that can be studied e.g., by perturbations, gives the researcher information on where and when specific parts of the system should be studied [22]. Another systems biology approach is the physics-inspired top-down modelling strategy, where features are identified of essential relevance to the phenomena of interest [21]. The less extensive model that is achieved is often combined with available data. If data is available, both quantitative and qualitative predictions can be made, however, if data are sparse or rare, then qualitative studies are accessible (figure 2). The two approaches towards a holistic view of the biological phenomena under study differ mainly by their complexity, in the sense of assessable information available to estimate the network and the numerical values of model parameters. The idea is similar to what is done in integrative physiology, where a biological question is studied on different scales at different levels of detail, either from cell-free systems (bottom-up) or from animal or clinical studies (top-down).

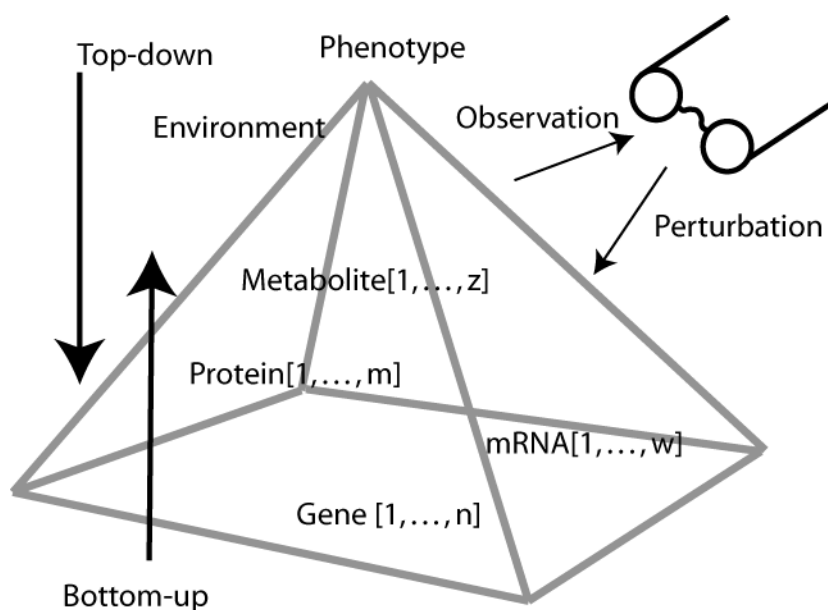


Figure 2. The bottom-up versus top-down view. An object can be studied of an observer from different perspectives. In the bottom-up strategy, the details are assumed to be important and this allows the observer to draw conclusions about the function of the system. In the top-down approach, the details are not important for the systems overall behaviour.

1.3.2 General assumptions

The law of mass-action is obeyed in the description of chemical interactions by the top-down, as well as the bottom-up strategies. This is an essential assumption that implicitly states that on the small metric-scale, diffusion is the only physical mechanism that works. In the small-scale-world, stochastic effects matter, such as molecular systems, however, as the number of specific molecules increases, a

deterministic model can be considered. In a deterministic model, it is assumed that the average effect is essential [23].

1.3.2.1 Set of ordinary differential equation versus stochastic differential equation

A popular and commonly used approach to describe molecular interactions is by a set of ordinary differential equation (ODE) [24, 25]. In ODE, time is considered as the independent variable. In a system of ODE, space is discarded; however, using compartments, it is possible to model space. The use of spatial differential equations (PDE) allows modelling interactions in space, whereas both time and space are independent variables. Mathematical models based on ODE are typically easier to analyse [26] than PDE, and this is one of the main reasons why ODEs are preferred.

1.3.2.2 Molecular network

The molecular network is crucial, as it can determine the qualitative behaviour of the system [27]. If the network is known, then the qualitative behaviour is dependent on the parameters values. However, if the network is unknown, either partially or entirely, then the modelling identification can predict the network. The predictions have to be confirmed by experimental studies.

An un-supervised strategy to assess a network would be to measure time-courses of the molecular entities or processes of interest, supervised from verbal models, and then use optimization methods that automatically will predict the network interactions and the complexity of the single functions used [28]. The quality of the predicted network will then be dependent on the available data, and not by subjective interpretations of data.

1.3.2.3 Model parameters

To parameterise bottom-up models, the input needs to be informative in the sense of resolution, both temporal and spatial. Preferably, the experimental design should allow for measurement of data on single components in dense time-courses, repetitively. This is not the way most data is available today, instead data is presented as mean values or even as relative data. This restricts the applicability of dynamical mathematical models, even though many fruitful models have been developed with these limitations. Top-down models can be easier to parameterise, as the need for detailed measurement is less. However, it is important that the entities in the model are designed in a measurable way.

Beyond the ability of dynamical mathematical models to qualitatively describe steady state behaviour, they have the power of describing transient behaviours as well. To do so, temporal and quantitative data is preferable.

1.4 HOMEOSTASIS

Homeostasis is central in physiology [29], as the mechanism of maintaining a functional and responsive organism in a changing environment is essential for survival [30]. A definition of homeostasis is; maintenance of the system at a set-point given a specific environment. It is important to understand that homeostasis is not about maintaining a constant level of, for example, glucose in the circulation; it is to bring back the varying concentrations of glucose to a set-point that suits the actual

situation. This regulatory system can be designed in several ways [29]. However, the existence of negative feed-back-loops is essential [27]. If the internal regulatory systems is altered, or defect, a certain change in the environment may cause dys-regulation. Such a change can be the situation that the human population faces in the developed countries, where over feeding and a sedentary lifestyle has been adopted [31]. These patterns of behaviour are hypothesised to be one of the main causes in the metabolic syndrome [31, 32].

1.4.1 An alteration of the glucose homeostasis set point: Diabetes Mellitus

Diabetes mellitus is a disease where the concentration of glucose in the circulation is increased due to an inability of insulin to lower the glucose concentration. Diabetes mellitus is divided into several sub-groups, where Type 1 is mainly caused by an auto-immune response [33, 34], mature onset diabetes in young (MODY) is caused by a group of single gene mutations that are severe and causes diabetes in young people [35-37], Type 2 (T2DM) is a disease that mainly has its onset in middle aged and elderly people, however a sedentary lifestyle, can in combination with specific genetic alterations increase the risk for the development T2DM in younger ages [38-41].

Irrespective of the type of diabetes mellitus, the incidence worldwide is increasing dramatically according to the International Diabetes Federation (IDF) (<http://www.idf.org>). The diagnosis of T2DM is often preceded by impaired glucose tolerance (IGT). According to the IDF, IGT is defined when the plasma glucose, two hours after consuming a 75 g glucose load, is greater than 7.8 mM (normal level), but remains lower than 11.1 mM (diabetes level). The level of plasma glucose is measured by means of an Oral Glucose Tolerance Test (OGTT). Fasting plasma glucose levels than 7.0 mM are above normal, but below the threshold for the diagnosis of diabetes. The ability of insulin to regulate the blood glucose levels is impaired in T2DM. To understand this dys-regulation, an increased understanding of glucose homeostasis is required.

The main insulin responsive organs are skeletal muscles, adipose tissues and liver [42-44], (figure 3). The regulatory mechanism in the responsive tissues that controls the homeostatic glucose concentration is mainly regulated by insulin and glucagon [43, 45, 46]. The pancreatic β -cells in the islets of Langerhans increase the insulin secretion in response to an elevation in the concentration of circulating glucose [47]. The pancreatic α -cells, also located in the islets of Langerhans secrete glucagon in response to hypoglycaemia [48, 49]. Glucagon increases glyconeogenesis in liver [50, 51]. Insulin decreases glyconeogenesis and increases glucose uptake in its responsiveness tissues [46, 52-56]. Thus, this regulatory system is driven by the deviation of glucose from its homeostatic set-point in the circulation, by negative feedback. The modulation of glucose uptake by insulin to maintain glucose homeostasis in the circulation is affected by regulatory mechanisms in the responsiveness tissues. Hereafter, only the mechanisms in skeletal muscles will be considered.

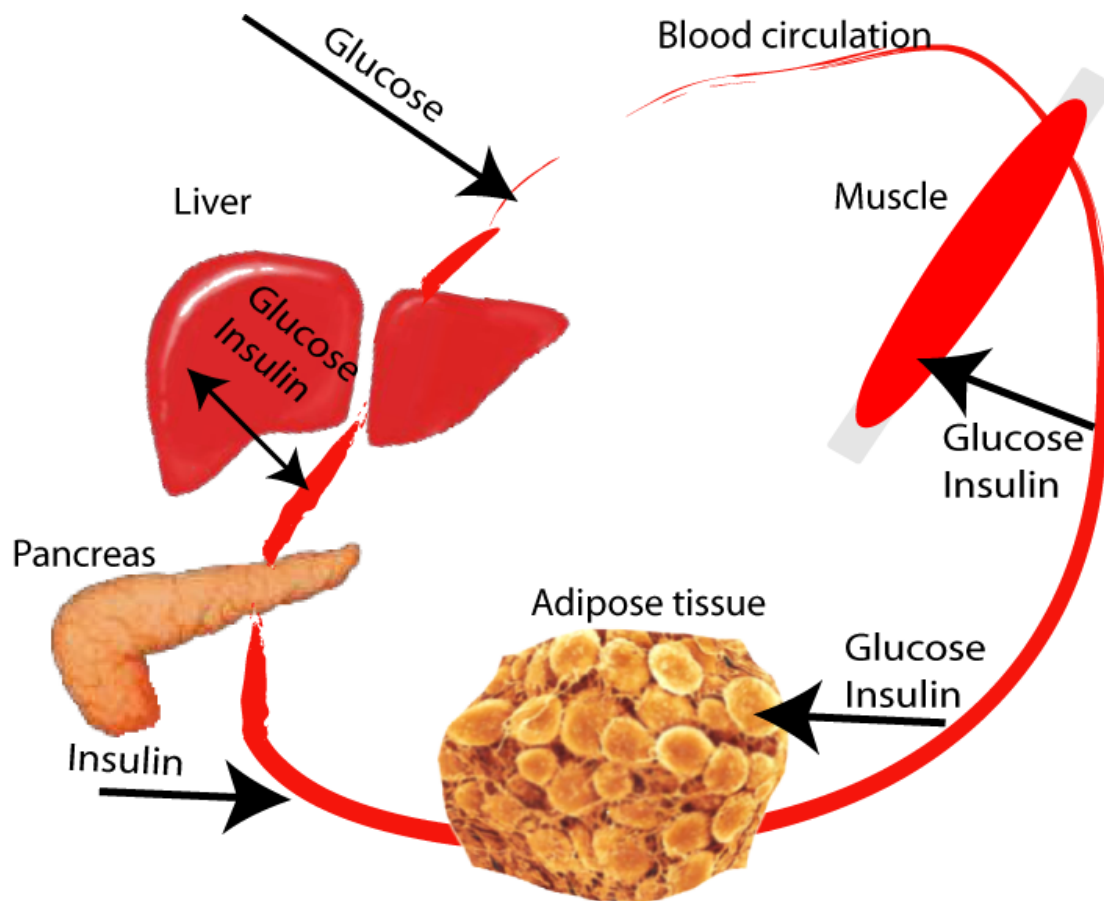


Figure 3. Insulin sensitive organs. A post-prandial rise in the glucose concentration triggers the pancreas to secrete insulin into the circulation. The secreted insulin increases glucose uptake into adipose tissue and skeletal muscle and inhibits hepatic glucose output.

1.4.2 Muscle Physiology

There are two classes of muscles, smooth and striated; striated muscle is further divided into heart and skeletal muscle. Smooth muscle surrounds blood vessels and the gastrointestinal tract. Skeletal muscles, which is the focus of this thesis work, is controlled by the somatic nervous system and hormones.

Skeletal muscle is made up of different fibres (figure 4). The fibres are formed by myoblasts that fusion into multinucleated myofibres [57]. The fibres are traditionally divided into three categories, type I and type IIA and IIB fibres, based on immunohistochemical staining of biochemical characteristics [58-60].

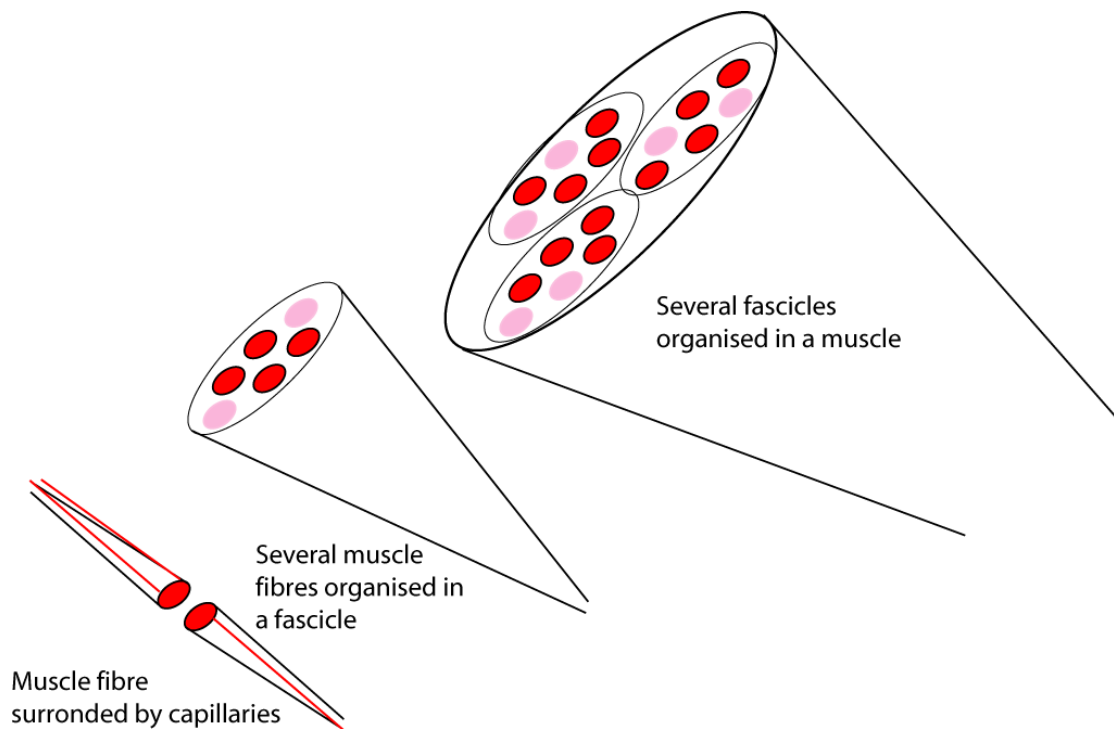


Figure 4. The structure of a skeletal muscle. The muscle is built up of several similar fibres, into fascicles, which are then grouped to build-up the muscle. The fibres are different on the small scale, i.e., metabolic profile, signalling capacity etc. Different muscles are as well predominantly built up of different fibre types [61-65] and finally, the fibre types are distinctly distributed within one muscle [63-65]. The more oxidative fibres are always more centrally located in the muscle tissue [62-65].

1.4.2.1 *Skeletal muscle fibre classification*

One fibre type can be classified into adjacent fibre types depending on the method used [60]. The classification is done by accessing different characteristics of muscle fibres. The use of myosin heavy chain specific antibodies [61] or myosin ATPase [58-60] staining determines the contractile phenotype of fibres. The use of succinate dehydrogenase staining reveals the oxidative capacity of the fibres, as it stains a mitochondrial enzyme.

1.4.2.2 *Skeletal muscle fibre plasticity*

Evolutionary forces are hypothesised to minimized the energy costs for maintenance and maximize the functionality of skeletal muscle tissues for all species [66]. The plasticity of the skeletal muscles transcriptome, and hence the proteome, allows the animal species to adapt its phenotype to changes in the environment on a short time scale. The contractile profile adaption demands a prolonged alternation in the use of the muscle [66]. The metabolic profile changes after acute exercise of the muscle, however, the mRNA level returns to its baseline within 24 hours [67]. Furthermore, in the context of glucose homeostasis, insulin signalling capacity is dependent on the fibre type [68], *paper 3*.

1.5 REGULATION OF METABOLIC AND SIGNALLING CASCADES

1.5.1 Summation theorem

The control over a pathway, independent of whether it is metabolic flux of e.g., glucose metabolites or a signalling cascade, is defined by the summation theorem [69, 70]. The summation theorem is derived from Eulers theorem for homogeneous function of degree one [71]. In the metabolic control analysis, the summation theorem has a central role [72], and has been useful when analyzing the control over a pathway. The summation theorem implies that the overall control of the fluxes in a pathway is a property of the entire pathway and not a characteristic of single components.

1.5.2 Rate limiting step

In the analysis of the control steps of a pathway e.g., to identify drug targets, the rate limiting step is of major concern [73, 74]. But, the control is always distributed among all the components in the pathway [75], as implied by the summation theorem. The step with the largest numerical value is considered to be a rate limiting step [73-75]. However, which component in the pathway that is the rate limiting step, depends on the actual situation, since the pathway is a dynamical entity. Generally, the initial step and the branching points in a pathway are often the steps that have the major control over the entire pathway [76, 77].

1.5.3 Insulin-dependent glucose uptake

The permeability of the plasma membrane to glucose is low [78, 79], due to the chemical characteristics of both the lipid layer and glucose itself. Therefore, a specific mechanism is required for allowing glucose to penetrate across the cell membrane, (figure 5). This mechanism was initially proposed to be a channel that was opened by the influence of insulin [78]. Today, it is known that insulin promotes glucose uptake by increasing the amount of glucose transporter 4 (GLUT4) at the plasma membrane [80-86]. The facilitated diffusion of glucose that occurs after GLUT4 has been incorporated to the plasma membrane increases the intra-cellular concentration of glucose. The amount of GLUT4 at the plasma membrane is considered as a rate limiting step for insulin dependent glucose uptake [82].

The molecular mechanism that regulates the translocation of GLUT4 is a switch mechanism [52, 81, 83, 87-90]. The circulating insulin concentration is the parameter that drives this switch [87]. The switch is further regulated by positive feedback loops via de-phosphorylation of the activated insulin/insulin receptor complex (AIRC) [27, 87]. The auto-phosphorylation that activates the AIRC in turn is due to positive-feedback, and works most likely by itself as a switch mechanism.

To maintain the gradient that allows glucose to enter the cytosol via GLUT4, irreversible phosphorylation of intra-cellular glucose to glucose-6-phosphate (G6P) occurs [91] (figure 5). The G6P is negatively regulating hexokinase II, an enzyme that is responsible for the phosphorylation of intra-cellular glucose [92, 93]. Furthermore, G6P is involved in the regulation of glycogenesis [91, 94]; hence, G6P is a master sensor for the intra-cellular glucose derivate status.

The insulin signalling cascade is believed to be defect in IGT and T2DM [93, 95, 96]. The actual defective place is not entirely known, even though data indicates that genetic alteration of the insulin receptor substrate 1/2 (IRS) impair the

transduction of the signal [97]. The binding of IRS to the AIRC is a regulatory step that seems to decide whether the signal will be further processed or not [97, 98]. The auto-phosphorylation of insulin receptor that occurs after insulin binding [99] triggers internalisation of the bound complex into the endosomal pathway [100, 101]. Insulin signalling is hypothesised to continue during this process until de-phosphorylation occurs [101-104]. Defective insulin signalling decreases the amount of GLUT4 at the plasma membrane [82, 105-107], causing a decreased diffusion capacity for glucose; hence, the increased concentration of glucose may occur in the circulation and cause IGT or T2DM. The capacity of different skeletal muscle fibre types to execute insulin-dependent glucose uptake varies [68], *paper 3*. The fibre type plasticity may directly affect whole body insulin-dependent glucose uptake [107]. The increased glucose taken up into the cytosol is mainly stored as glycogen [91], if not used to produce energy.

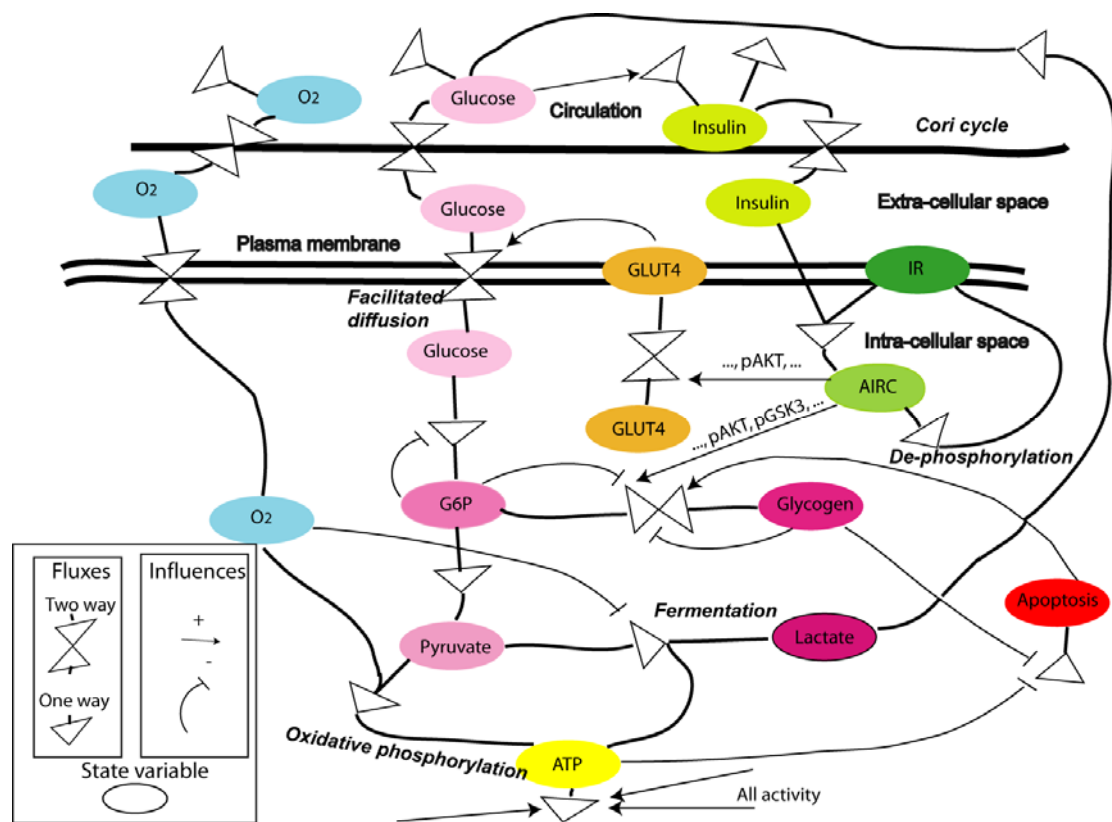


Figure 5. Cartoon model of the effect of insulin on glucose homeostasis. The cartoon model can be translated into a mathematical model in an unambiguous way, by applying the law of mass action on all interactions. In the cartoon, three different sets of symbols are used to describe all interactions; the flux-symbols, the influence-symbols and the symbol for state variables. The fluxes can either be one or two directions and the influences can be positive or negative. There is always a positive arrow from the state variable into flux that leaves a state variable. This arrow is not drawn. The fluxes are typical molecular processes and can be expended (more bottom-up approach) or condensed with state variables (more top-down). This cartoon model has four compartments, intra-cellular, plasma membrane, extra-cellular and circulation. In the process of transform the cartoon model into a set of differential equations, consideration should be taken to the time delay that are in the Cori cycle and the interaction between glucose levels in the circulation and the release of insulin from the pancreatic β -cells. For the description of the molecular interactions see the paragraphs under 1.5. The same component in different compartments is assigned different colours, and derivatives are colour-coded within the same colour-scale.

1.5.4 Insulin-dependent glycogenesis and glycogenolysis

Insulin signalling affects glycogenesis [91, 94, 108] by specific phosphorylation of glycogen synthase kinase on serine 21/serine 9 (GSK3) via Akt on serine 473 (figure 5). The phosphorylation of GSK3 on serine 21/serine 9 increases the ability of glycogen synthase (GS) to amplify the glycogen chain [99, 109, 110] by incorporating glucose derivatives into glycogen. The GS is an allosteric regulated enzyme where the main regulators are G6P and glycogen [91, 111]. An increased concentration of G6P increases the rate of glycogenesis, where a high content of glycogen inhibits the rate [111] (figure 5). These two molecular components are assumed to have the major control on the rate of glycogenesis [108]. Hence, insulin action is sufficient, but not essential to increase the rate of glycogenesis.

The local glycogen concentration within a muscle fibre regulates the response to insulin [108, 112, 113]. Large glycogen storage pools inhibit glucose uptake [113]. The molecular mechanism can be either the pathway effect that via glycogen inhibition of GS affects the ability to incorporate more glucose derivatives into glycogen or by inhibition of a key step in the insulin signalling cascade via reduced phosphorylation of Akt [87] (figure 5).

1.5.5 Glucose utilization for energy production

Glucose derivatives can either be oxidised or fermented to produce energy. The main difference is the efficiency of these pathways. Oxidative phosphorylation gives approximately 15 times more energy, as ATP, from each glucose molecule than fermentation. Lactate is produced as a temporal local end-product after fermentation, which is re-circulated to the circulation as glucose via the Cori cycle. Oxidative phosphorylation is dependent on the availability of oxygen and takes place in the mitochondrial matrix (figure 5). The endpoint of oxidative phosphorylation is carbon dioxide, which is re-transported to the circulation and exchanged against oxygen in the lungs.

1.5.6 Anoxia effects on glucose uptake and utilization

Low intra-cellular levels of oxygen are defined as anoxia, whereas impairment in oxygen delivery to the tissues via the circulation is defined as hypoxia. The intra-cellular oxygen pressure is sensed by a molecular mechanism involving increased expression of the hypoxia induced factor alpha-1 (HIF1-alpha) upon reductions in the oxygen level [114-116]. The transcription factor HIF1-alpha increases many transcripts, including glucose metabolism-associated genes [117-121]. The outcome of this coordinated effect is increased glucose uptake and increased glycogenolysis [117-119, 121].

1.6 THEORETICAL MODELS IN SKELETAL MUSCLE PHYSIOLOGY

1.6.1 The top-down approach; Krogh's cylinder

In 1920, August Krogh was awarded the Nobel Prize in Physiology and Medicine, for his discovery of the capillary motor regulation. Through the measurement of oxygen diffusion into a muscle [122, 123] and the derivation of a mathematical model to answer the question on the minimal diffusion distance of oxygen from a capillary, he was able to explain how blood flow was regulated in the capillaries [123]. The Krogh

cylinder (figure 6) has been used by numerous researchers, see for example [124-126]. The major findings by Krogh were that instead of increased blood-flow through the muscle tissue capillary system upon exercise, which was the dominant theory at that time, increased flow was insufficient to meet the increased oxygen requirement of the organ, as the diffusion rate was too slow. Instead he proposed that an increased blood volume was required to meet the demand. He could show by a series of experiments that the number of capillaries that transported blood increased during exercise, and this increased blood volume, with unchanged blood velocity, supplied the muscle fibres with oxygen.

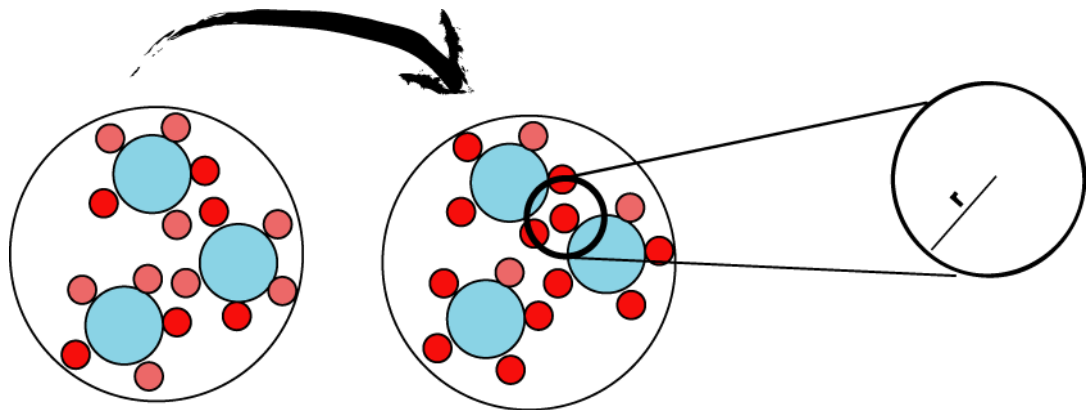


Figure 6. Krogh's cylinder. The velocity of blood fluid transport through a muscle is constant. To increase the amount of oxygen that can be taken up by diffusion, more capillaries open and allow for an increased flux of blood through a muscle. This mechanism makes the fluid velocity constant, and, hence, the diffusion of oxygen is rapid enough to meet the demand of the working muscle. Krogh's cylinder describes the minimal distant, r , between two adjacent capillaries in a muscle, which are required to guarantee that intra-cellular oxygen pressure is maintained during a specific perturbation. The muscle fibres are drawn as blue circles, the red and pink circles are drawn to show capillaries either open or closed, respectively. The oxygen consumption rate increases with exercise, as indicated by the black arrow. The Krogh cylinder cartoon model is drawn at the right.

1.6.2 The top-down approach; minimal diffusion distance

In 1928, Archibald Vivian Hill [127] presented a mathematical model describing the diffusion of oxygen into a muscle preparation during steady state conditions. He discussed diffusion on the scale of $\mu\text{m}^2/\text{millisecond}$. The rapid diffusion attainable in a system of small dimension is the basis of the capillary system. Hill was interested in the limitation of the experimental method of *in vitro* incubation of skeletal muscle specimen, which he had used to determine the heat production during contraction and recovery [128], for which he was awarded the Nobel Prize in 1922. To better understand the diffusion of oxygen into the muscle specimen and lactate release from the same muscle, he derived a mathematical model that assumed homogeneous metabolism, and that the critical value for oxygen pressure was equal to zero. The solution of the mathematical equation gave a minimal diffusion distance that could be obtained by assuming different consumption rates of oxygen (figure 7).

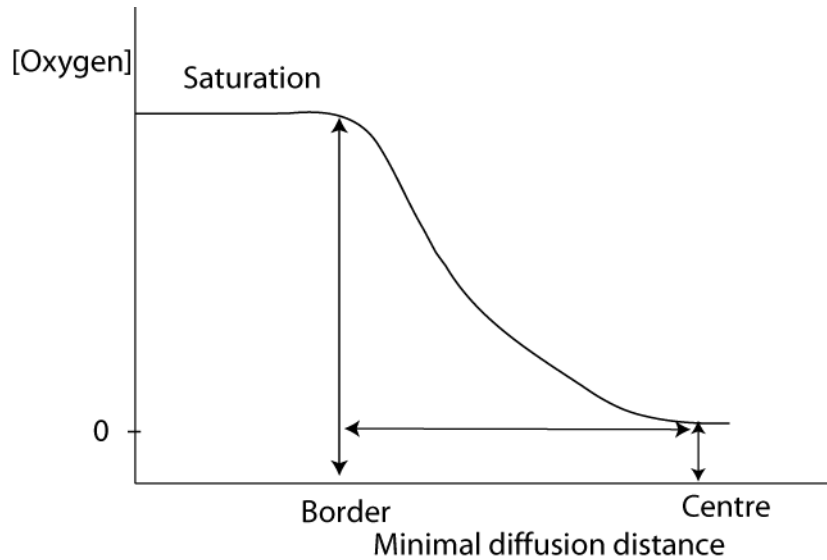


Figure 7. The definition of the minimal diffusion distant (adapted from Hill [127]). The null-isocline was used as the critical value of intra-muscular oxygen pressure. The intersection between the calculated gradient of oxygen content and the null-isocline defines the minimal diffusion distance.

1.6.3 The bottom-up approach; insulin effects on glucose uptake

Skeletal muscle glucose uptake and utilisation are studied to better understand the mechanism that regulates glucose homeostasis, as skeletal muscles is the major post-prandial deposit for glucose [129]. Altered regulation of glucose homeostasis is a factor leading to the onset of T2DM. Mathematical models have been developed to address the effect of insulin on glucose uptake, however, as experimental models, either data from adipocytes or whole body studies are used. Even though, the network may be considered to be representative, the parameter values might not. It is not clear if skeletal muscle specific parameters would give different qualitative results. However, the model developed by Sedaghat *et al.* [130], pointed out that a negative feedback loop could explain previous data [131] on protein kinase C- ζ dynamics. Further analysis of Sedaghat's model by Kwei *et al.* [22] revealed that a more mechanistic model is required to facilitate the understanding of insulin effects on glucose uptake. Furthermore, Kwei *et al.* analysed the minimal number of state-measurements that were required to identify all of the parameters in Sedaghat's model. They concluded that more realistic time-course measurements and an increased number of samples would improve the model identification. In another study by Kwei *et al.* [132], the authors transformed Sedaghat's model into a stochastic model, due to the fact that when they rescaled the model to take into account the volume of one adipocyte, many components were found to be in low numbers, i.e., under one hundred. Further analysis revealed that denser time-course measurements are required, preferably on single-cells, in the first minutes after the addition of insulin, when signalling events start.

A steady state model was developed by Giri *et al.* [87] that focused on the impact of feedback loops on the qualitative behaviour of GLUT4 at the membrane. Experimental observations indicate that the translocation of GLUT4 to the membrane is a switch-like mechanism [52, 81, 83, 87-90]. Analysis of steady state behaviour of Giri's model shows that a hysteresis effect causes the translocation of GLUT4 upon insulin stimulation [87](figure 5).

1.7 QUANTITATIVE DATA FROM IMAGES

The highest quality of quantitative data would be the actual numbers of molecules or entities. However, this is seldom achieved; instead concentration (mol/L) is used, which can be transformed into numbers by using the Avogadro constant. The relation between a measured concentration and the number of molecules is not always linear, as assumed when using Avogadro's constant. In reality, it is only in diluted solutions that the measurement of a concentration is reflecting the number of the components [133].

The process of obtaining quantitative data from images can be obstructed by at least two circumstances; (1) the staining process and (2) the acquisition of the image. The processing and analysis of the acquisitioned images can be solved with known errors [134].

1.7.1 Immunochemistry

Staining can be performed by two principally different methods; using dyes or antibody-mediated staining i.e., immunohistochemistry. Staining with dyes occurs when a compound interacts with the molecule of interest and increases their visibility. In *paper 2*, Periodic-acid-Shiff staining (PAS) was performed, which is a method that uses dye. Antibody-mediated methods can be divided further into enzymatic immunohistochemistry via biotin or immunofluorescence via fluorophores. The enzymatic immunohistochemistry technique requires a second step, where the enzyme is added and a coloured product is formed that can be detected using a light microscope, (figure 8). The staining produced will not detect small particles, as the dye has a tendency to flow out and cover a bigger area than the one covered by the actual source. The same phenomenon is achieved when dyes are used. Both of these methods are easy to handle and less expensive than fluorescence-based methods. Immunofluorescence, (figure 8), on the other hand, can detect single molecules in living cells [135-137], which is beneficial. To do so, the background fluorescence should be avoided by using appropriate chemicals [135-137]. To get high qualitative quantitative data, the use of both primary and secondary antibodies should be performed to avoid any unknown signal amplification (figure 8). This is actually one of the limiting steps to obtain the highest quality of quantitative data from immunofluorescence techniques. Immunofluorescence was applied in *paper 2 and paper 3*. Furthermore, a double staining procedure was applied in *paper 2*, where PAS-staining and immunofluorescence techniques were combined.

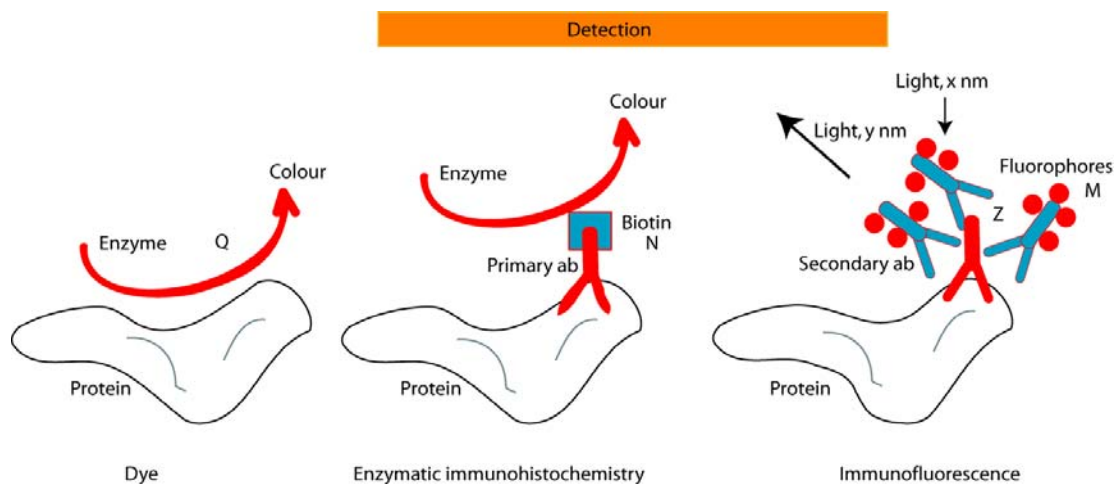


Figure 8. Three different principles to detect target molecules in cells. Left shows staining with a dye that allows for the detection of the molecule of interest. Middle shows the use of primary antibodies coupled to a signal enhancer, Biotin, which allows for the detection. The signal amplification is (Q, N) unknown for both these cases. Right shows that the detection of emitted light from single proteins by immunofluorescence is technically feasible, however, the unknown signal amplification (Z, M) is a limitation when higher amount of protein are to be detected. Q = signal amplification when dyes are used, N = signal amplification when Biotin is used, Z = signal amplification between primary and secondary antibodies and M = signal amplification of fluorophores binding to the secondary antibody.

1.7.2 Quantitative image analysis and processing

The visualization of single, purified molecules in aqueous conditions became available in 1995 [138]. This technique was then further developed such that single molecules could be detected in living cells in 2000 [135-137, 139]. Today, the technique is used to study structure, function, and the transition of the states of molecules [135-137]. The technique is, however, developed to study cultured cells. There are limitations that make it difficult to study tissue preparations, organs and whole organisms.

The processing of images deals with the computational handling of the acquisitioned images, whereas the analysis deals with the identification of objects and the creation of histograms from the underlying numerical matrix etc. In *paper 2* and *paper 3*, both image processing and analysis were performed. In *paper 2*, a quantitative image analyses method [140] was further developed to allow for statistical inference of the numerical matrix that build up the images acquisitioned. This method was then used in *paper 3*, even though the method is not entirely suitable for this purpose.

The benefit of having a quantitative method for image analysis is enormous, even though there are limitations in how quantitative image analysis can be mad. The quantification of images has certain limitations, both on the computational and the experimental side. On the computational side, most of the limitations are in the acquisition of images [134]. With good equipment that is handled correctly, most limitations will be prevented. On the experimental side, the actual staining process is the limitation for obtaining quantitative data in the sense of concentration. In *paper 2*, we avoid this limitation by using a standard curve that was acquired under the same conditions as during the acquisition of the actual data. However, this procedure does not yet work for images based on immunofluorescence. The advantage of obtaining high resolution 2-dimesional or 3-dimesional data is important, especially for the process of identifying a mathematical model.

1.8 EXPERIMENTAL SYSTEMS TO STUDY SKELETAL MUSCLE GLUCOSE HOMEOSTASIS

One conventional experimental model to assess insulin signalling and glucose metabolism in skeletal muscles is through the use of isolated tissues incubated *in vitro*, for a review see [141]. The overall methodology is similar to that analyzed by Hill [127]; however, the animal species is changed from frog to rat or mouse. The use of mouse tissues is advantageous, as the muscle samples are smaller in size compared to rat. Hence, the assumed limitations with diffusion would be less. However, the higher metabolic rate may interfere with the results. To minimise the diffusion distance, cell cultures would be beneficial. But, the use of cultured skeletal muscle (myocytes) to assess insulin effects is limited by the fact that GLUT4 is lowly expressed in the cultured cells. Therefore the GLUT4 response is minimal, even though the insulin signalling cascade seems to be intact with robust responses observed [142, 143]. To get an experimental system that allows for detection of both the insulin signalling cascade and a GLUT4 response skeletal muscle tissue is used. The use of *in vitro* incubation of skeletal muscles from rodents has been extensively validated previously [141, 144-147].

The experimental design allows control over the circumstances that might affect the incubated muscle specimens. Saturation of media that surround the tissues with oxygen can be a help to avoid anoxia [79, 141, 144, 148, 149]. Moreover, it is possible to incubate the muscle in a constant environment of glucose, insulin etc. Measurements can be done on components secreted into the media i.e., lactate and carbon dioxide, see method section. Furthermore, the muscle specimen is easily accessible for further studies of different omics.

A major concern with muscle preparation that is incubated *in vitro* is whether the entire specimen can be adequately oxygenated [127, 141, 145, 147, 148, 150, 151]. This concern has been addressed in at least two similar experimental settings, spherical cell cultures [150, 151], and in pancreatic β -cells [149]. The conclusion made in these studies indicates that oxygen does not diffuse into the experimental object in sufficient amount.

To ensure that the tissue preparation has unlimited access to oxygen, the incubation media is continuous gassed during the incubation time, moreover, the incubation buffers is also pre-gassed with 95% O₂ and 5% CO₂ [79]. This procedure guaranties that the O₂ is saturated within the media. Even though, the saturation of the media may be secured, the incubated specimens might not [149-151]. The assumption that the incubated muscle specimen is sufficiently oxygenated has been tested several times with different techniques [127, 141, 148], and is not consider as a major drawback, however, the glucose metabolic data obtained is incompletely understood [152]. There are two specific molecular processes measured that do not follow the expected outcome.

Glycogen content after insulin stimulation is not always increasing [153, 154]. This phenomenon has been hypothesised to arise from limitations in the analytical methods used [152]. To circumvent this limitation, biochemical measurements are performed to assess the rate of glucose incorporation into glycogen during a specific time period. The amount of glucose incorporated into glycogen is then used as an estimate of glycogen synthesis. The next molecular process that is incompletely understood is the observed high rate of lactate released from the incubated specimens, with or without insulin stimulation [152], *paper 1*. This observation is interpreted as an additional mechanism in glycolysis that works as a safety valve [152].

2 AIM OF THE THESIS

The overall aim of this thesis is to combine mathematical modelling with existing biochemical data and knowledge to determine whether a holistic approach would increase the understanding of mechanisms controlling the regulation of glucose homeostasis in skeletal muscle.

- Derive a mathematical description of glucose metabolism upon insulin stimulation as it appears in the isolated incubated muscle specimens.
- Validate predictions from the newly derived mathematical model on anoxia as a cause for glycogen depletion after incubation of isolated skeletal muscle specimens.
- Determine whether insulin diffusion is sufficient to trigger its signalling cascade within the incubated muscle specimens.

3 METHODS

3.1 SYSTEMS OF ORDINARY DIFFERENTIAL EQUATIONS

Properties, such as the dependence on time and space in biological systems, are important to characterise. When both temporal and spatial parameters are essential, partial differential equation is appropriate. However, if only one dimension is considered, then ordinary differential equation (ODE) is satisfactory. The general form of a system of ODE is:

$$x'_i = f_i(x_1, \dots, x_n, p_1, \dots, p_l, t)$$

Where x' denotes the time (t) derivative, f_i an optional function $i = 1, \dots, n$, x_i denotes variables, e.g., concentration, p_i denotes model parameters. The model-parameters are preferably determined by data.

3.1.1 Model complexity

The complexity of the selected mathematical description is dependent on several decisions;

1. the scientific question
2. bottom-up *versus* top-down
3. which interaction level is considered e.g., interaction between molecular events or tissues, or both
4. the availability of data and the scale and resolution that these are on, e.g., transcriptome, proteome, metabolome etc, and if it is steady-state or time-course
5. the qualitative behaviour assumed, e.g., is phenomenological expressions such as Michaelis-Menten kinetic applied or is interactions described mechanistically.

In the mathematical model presented in *paper 1*, data from glucose metabolism was available on the scale of whole muscles from steady-state measurements. Hence, the model complexity is described as one compartment (OCM). The time-scale on which enzymes work is on the millisecond to second scale, and data is collected after fifty minutes of stimulation, any changes in enzyme activity has then been stabilized, and hence, a zero and first order expression is enough to describe the dynamics of glucose utilization close to steady state, *paper 1*.

3.1.2 Mathematical analysis

To analyse the global behaviour of the mathematical model in *paper 1*, stability analysis was performed. To determine the stability of the OCM we used the analytic solutions of the system. The general solutions of the system of ordinary differential equations were analyzed to judge the behaviour of the system within the experimental time-frame.

3.1.3 Estimation of parameters and constants

The estimation of parameters in *paper 1* was performed using the data sets median value. For analysis of the estimated concentration of G6P's impact on the system, qualitative and quantitative behaviour was performed. The parameters (k_i) are all non-negative.

$$k_i \geq 0$$

3.1.3.1 Sensitivity analyze

3.1.3.1.1 Single parameter perturbation

Sensitivity analysis was performed to examine the response of the system to changes in the parameters. For a system of ordinary differential equations, perturbations of a parameter p_i may cause a change in variable x_i .

$$S_{ii}(t) = \frac{dx_i(t)}{dp_i}$$

A sensitivity analysis was performed with the software MATLAB, (MathWorks, www.mathworks.com).

3.1.3.1.2 Two parameter perturbation

To investigate the influence of several parameters simultaneously, a combined perturbation of two or more parameters can be performed. In *paper 1*, a combination of two-parameter analysis with the available data was performed using MATLAB, software (MathWorks, www.mathworks.com).

3.2 QUASI STEADY STATE

The OCM in *paper 1* was in quasi steady state (see result and discussion). This feature was used to reduce the model into a single differential equation that was solved explicitly. The reduced model can be combined with the quantitative spatial measurement of glycogen, *paper 2*, to estimate the single-fibre concentration of glycogen, and to estimate the rate of glucose uptake in the superficial fibres.

3.2.1 Empiric cumulative data distribution function

An empirical cumulative distribution data function (ECDF) was created (figure 9) from data in *paper 1*. The ECDF was then used to investigate the impact of the median on the model prediction. One hundred simulations were performed to avoid over interpretation of the results.

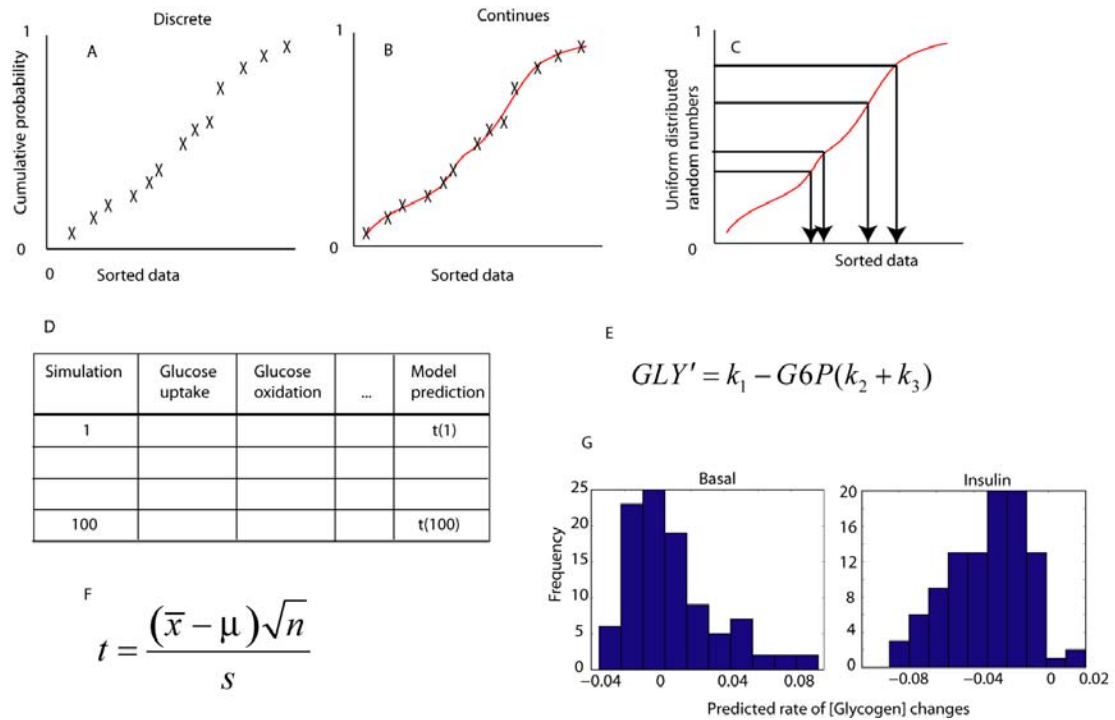


Figure 9. The construction of an empirical cumulative distribution data function (ECDF). One monotonic increasing continuous function (B) was derived from each sorted data set (A). One hundred uniform distributed numbers between 0 and 1 were randomly drawn. Each ECDF was then used to transform the random numbers into new virtual experiments (C). The new data points were further used to calculate the new parameter values. Model simulations (E) were performed for each data set (D). The model prediction was then analysed with a Student t-test to judge if the predictions were distinguished from zero, basal state (two-sided t-test), or larger than zero (right-sided t-test) for the insulin-stimulated state (F). The frequency of each model prediction was visualized in a histogram to be accessible (G).

3.3 METABOLIC MEASUREMENTS ON SKELETAL MUSCLE AFTER *IN VITRO* INCUBATION

3.3.1 Animals

We have focused on data from basal and insulin-stimulated states mouse *extensor digitorum longus* (EDL) muscle samples obtained from a cross between CBA and C57Bl/6J wild-type mice [155]. Mice were maintained in a temperature- and light-controlled environment and had free access to standard rodent chow and water. Mice were anaesthetised with Avertin (2,2,2)-Tribromo ethanol 99% and Tertiary amyl alcohol (0.015-0.017 ml/g of mouse body weight) and the EDL muscle was rapidly dissected and submerged into an oxygenated solution Krebs-Henseleit bicarbonate buffer (KHB) at 30°C. The local animal ethical committee approved all experimental procedures.

3.3.2 Muscle incubations

The incubation media was composed of KHB containing 0.1% bovine serum albumin (RIA grade) [156]. Media were continuously gassed with 95% O₂/ 5% CO₂. Muscles were incubated in a recovery solution (10 minutes) containing KHB and 5 mM glucose and 15 mM mannitol. Thereafter, a pre-incubation step occurred in the absence or presence of insulin (12 nM) (20 minutes) in the KHB solution. Next, the muscle was rinsed in 20 mM mannitol with no glucose (10 minutes) in the KHB solution. In the last incubation step, the hot-incubation, radio-labelled glucose was included to metabolic

determinations, but not for staining purpose, where unlabeled glucose was used. During this last step (20 minutes), metabolic data was collected. This step was different between each experiment and depended upon each incubation protocol. The total incubation time was 60 minutes for all experiments, whereas in the last 50 minutes, muscles were incubated in the absence or presence of insulin.

3.3.3 Glucose uptake and lactate release

In the hot-incubation, muscles were transferred to KHB containing 1 mM 2-deoxy-[1,2,3H]glucose (2.5 μ Ci/ml) and 19 mM [14C] mannitol (0.7 μ Ci/ml) and incubated. Glucose uptake was expressed as mM per litre (L) of intracellular water per hour [156]. For the assessment of lactate release muscles were transferred into KHB containing 5 mM glucose and 15 mM mannitol and incubated for 20 minutes (the hot incubation). The media was thereafter collected and lactate concentration was measured using a lactate assay kit (Biomedical Research Service Centre, University at Buffalo).

3.3.4 Glucose oxidation and glucose incorporation into glycogen

The glucose was supplemented with [14 C]-glucose (0.2 mCi/ml) and muscle were incubated for 60 minutes. Thereafter, 0.2 ml of SolvableTM (2% Sodium Hydroxide; DuPont, Hamburg, Germany) was injected into the centre-well of the incubation vial for the collection of liberated CO₂, and 0.5 ml of 15% perchloric acid was injected into the media for acidification. Glucose oxidation was assessed by collection of liberated [14 CO₂]. Rate of glucose incorporation into glycogen was assessed by incorporation of 14 C into glycogen. Muscles were homogenised in 0.5 ml of 1 M NaOH and subsequently 0.5 ml of 20% trichloroacetic acid was added. The homogenates were centrifuged for 15 minutes at 3500 x g and glycogen in the supernatant was precipitated by addition of 200 μ l of 110 mM glycogen and 2 ml of 95% ethanol. The glycogen precipitate was then collected by centrifugation at 2000 x g for 15 minutes and dissolved in water for liquid scintillation counting.

3.3.5 Glycogen content

Skeletal muscle (4-10 mg) was homogenised in 0.5 ml 1M HCl at 100°C for 1 hour. Glycogen was then measured fluorometrically as described previously [156].

3.4 IMMUNOHISTOCHEMISTRY

3.4.1 Cryostat sectioning

Frozen muscle samples were mounted with a drop of OCT compound (Tissue-Tek, Sakura Finetek, NL) on pre-cooled and pre-holed cork plates. A thin layer of OCT was created to give the muscle support during the cryosectioning process. Sections of 14 μ m were created on a Microm HM 500M -23°C, mounted on SuperFrost (Menzel GmbH & Co.) microscopic slides and stored at -20°C until use.

3.4.2 Quantitative immunohistochemistry

3.4.2.1 Glycogen measurement

Muscle glycogen was analysed as previously described [140]. A glycogen standard was used as a reference to estimate the glycogen concentration in the muscle sections. Several 4% gels (0.125 M Tris, 13.0% crylamide (30:0.8%), 1.9% APS, 1.0% TEMED) were cast with a standard of known glycogen concentration. Gel specimens (~2 mm) in diameter, were isolated and frozen in OCT and sliced in 14 μ m sections

and subjected to PAS staining. A glycogen standard was prepared (Sigma G8751-5G; 0.200, 0.150, 0.100, 0.050, 0.025 and 0.000 M (blank)) (figure 10). The standard curve was non-linear. The function for the standard curve was used when the samples were quantified.

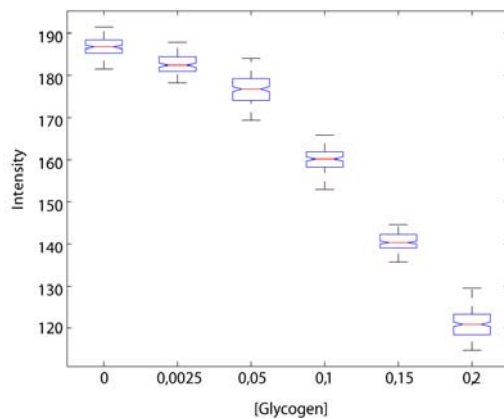


Figure 10. Boxplot of glycogen standards. A standard curve was derived from the median values of measurements on the glycogen standard. The acquisition of the images was performed with the same settings, microscopy and camera, as for the muscle samples.

3.4.2.2 Periodic-acid-Shiff (PAS) staining

Muscle glycogen was stained using a standard PAS protocol as follows: muscle sections were fixated for 1 hour at 48°C in 3.7% (v/v) formaldehyde in 90% ethanol, pre-treated for 5 minutes with 1% (v/v) periodic acid, washed for 1 min with water, wash-dipped for 5 seconds in dH₂O, incubated in 1% Schiff's reagent for 15 minutes at room temperature, washed 5 seconds in dH₂O followed by 10 minutes in water with gentle agitation, and finally, washed five times in PBS. The slides were dehydrated 37°C for 30 minutes and the cover glass was mounted with Mountex (HistoLab Products, Sweden). To visualise the stained muscle sections, Axioscop2MOT (Carl Zeiss AB, Sweden), 2.5 was used with an Olympus DP70 camera (Olympus AB, Sweden) and CAST 2.3.1.6 software.

3.4.2.3 Immunofluorescence

In *paper 2*, immunofluorescence was used to detect HIF1-alpha (sc-10790) and caspase-3(sc-7148) content. In *paper 3*, detection of specific phosphorylation of the insulin receptor (IR) on tyrosine 1146, Akt on serine 473 and GSK3 on serine 21 and 9 was determined. The Zenon Alexa Flour 555 rabbit IgG labelling reagent (Z-25305) was used (Invitrogen, Sweden). A slide containing the frozen tissue section was thawed at room temperature for 15 min. Muscle sections were rehydrated with PBS for 15 minutes in room temperature. The sections were permeabilised at room temperature using PBS containing 0.2% Triton X-100 (PBT) for 20 minutes. Nonspecific binding sites were blocked with PBT containing 1% BSA for 30 minutes at room temperature. Sections were incubated with antibody solution mixed in PBT for 2 hour. The staining solutions were removed and sections were washed in PBT three times for 15 minutes at room temperature. Sections were washed in PBS two times for 5 minutes. A second fixation was performed by incubating samples with 4% formaldehyde in PBS for 15 minutes at room temperature. Sections were washed one more time with PBS for 5 minutes. Thereafter, the sections were mounted using ProLong Gold anti-fade reagent with DAPI (Invitrogen P36931, Sweden). The concentrations used for the antibody versus the Zenon Alexa Flour 555 rabbit IgG labelling reagent was 1:6

(antibody/labelling reagent) and diluted 1:6 (antibody mix/PBT) for the working solution. Stained muscle sections were visualised using a confocal microscope (Inverted Zeiss LSM 510 META, Settings: Plane, multitrack, 12 bit, 1,024_1,024, 1,303.0 mm_1,303.0 mm, Plan-Neufluar 100/0.3).

3.4.2.4 Combined PAS and immunofluorescence staining

The following basic staining protocol with additions as previously described [140] was used for the combination of immunofluorescence and PAS. Briefly, a slide containing the frozen tissue section was thawed at room temperature for 15 minutes while borders were drawn with a PAP-pen (hydrophobic barrier pen for immunohistochemistry). Muscle sections were rehydrated with PBS for 15 minutes in room temperature. The sections were permeabilised at room temperature using PBS containing 0.2% Triton X-100 (PBT) for 20 minutes. Thereafter, the samples were treated as described for the immunofluorescence staining, except that the second washing step was performed twice.

3.4.3 Image analysis

3.4.3.1 Quantification of PAS-staining

The settings for the microscope and camera were equal for all images taken. No background extraction was made. Each image was rotated, using Photoshop, so that the border facing the left side of the image was positioned without defects. Three standardized regions (50 x 200 pixels) were selected from three cryosections taken in the middle part of each muscle, using MATLAB, (MathWorks, www.mathworks.com) (figure 11). To obtain a robust estimate of the mean intensity, all nine regions from one muscle were pooled together. An estimation of the spatial glycogen concentration was made using a standard-curve (figure 10). The matrix obtained was grouped to reflect the fibre sizes. The concentration values were normalized, either by the corresponding group in the control sample or by the first group, representing the superficial fibres (figure 11).

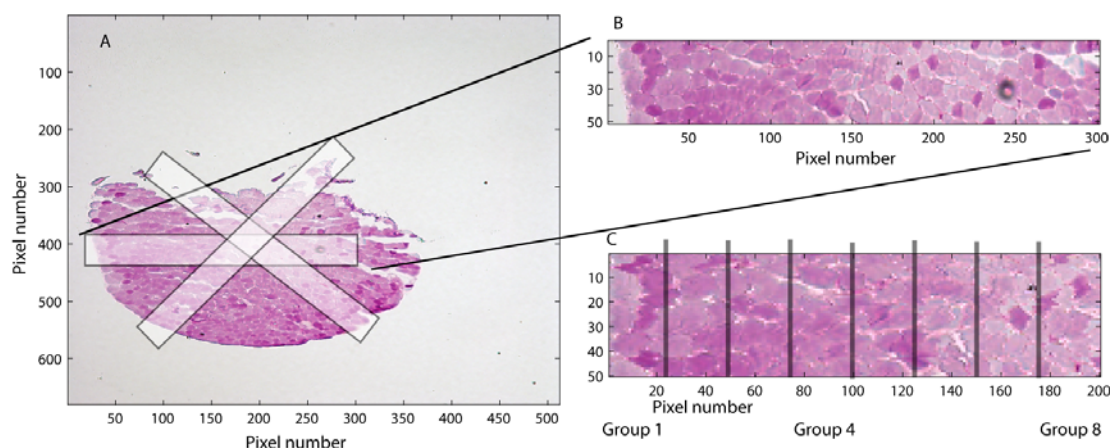


Figure 11. Selection, sorting and grouping of the image matrix. Each image was rotated with Photoshop, and then exported to MATLAB (MathWorks, www.mathworks.com), for handling of the underlying matrix. Three sections in the image, panel A, were manually selected, panel B. To avoid unnecessary variance due to any influence of the background, the section matrix was sorted so that the border of the muscle cryosection was aligned in the first pixel column, panel C. The mean of the group was then calculated to get a single row of data. To analyse the pattern, normalisation was done with the first group. To analyse the differences between the treatments, normalisation was done with the control for each group, *paper 2*.

3.4.3.2 Quantification of immunofluorescence staining

The images were taken via confocal microscopy, with equal settings for all images taken, and no background extraction made. The same procedure was performed for image processing as described for the quantification of PAS-staining (figure 11). However, the standardised region has higher amount of pixels due to increased resolution, otherwise the area was of similar size. The analysis procedure follows that described previously for the quantification of PAS-staining; however, the noise-to-signal ratio was too high in the images to allow statistical analysis of the results.

3.5 PHOSPHOPROTEIN ASSAY

The multiplex assay used detects phosphorylated insulin receptor on tyrosine 1361, phosphorylated Akt on serine 473 and phosphorylated GSK3 on serine 21 and 9. The analysis was performed according to the directions supplied with the commercial kit (Bio-Rad, Richmond, CA).

3.6 GENERAL STATISTICS

In *paper 1*, the data was analyzed using the Mann-Whitney U Test for differences between treatments. A confidence interval for the prediction of the model was obtained by simulating the model one-hundred times with random values from the empiric cumulative data distribution. A student t-test was performed to judge the outcome. In *paper 2*, a paired t-test and a Student t-test was used to test the outcome from image analyses. In *paper 3*, a paired t-test was used to analyse the phosphoprotein assay.

4 RESULT AND DISCUSSION

4.1 *IN VITRO* INCUBATION OF SKELETAL MUSCLE

The incubation of isolated skeletal muscle samples in an oxygenated solution is a commonly used method to study the *in vitro* hormonal response on metabolism [141]. The circulation is the physical mechanism that controls the delivery of oxygen and nutrients to each single muscle fibre. The blood in the circulation is under pressure, which causes advection of blood plasma within the tissue. In the experimental design for the incubation of muscle preparations, the circulation is interchanged with diffusion of compounds solvable in media into the muscle specimens. This major change may affect oxygen-dependent processes [79, 127, 144-151, 157, 158]. The effect of anoxia on glucose metabolism has been of major concern since the method started to be used almost one-hundred years ago [127, 141, 145, 147, 148]. As previously mentioned, Hill [127] investigated the minimal diffusion distance for oxygen within muscle specimens incubated *in vitro*. His results indicated that a positive intra-muscular oxygen pressure was present if the radial size was below 2 mm (figure 7). The following important assumptions were made; (1) the tissue was homogeneous and (2) the critical oxygen pressure was equal or above zero.

In the 1960's, a series of studies [78, 79] were performed to determine the effect of insulin on glucose uptake. The results laid the group-work for the discovery of glucose transporters over two decades later [80, 159]. The effect of anoxia on glucose uptake was also addressed [79]. The experimental design involved a comparison between muscle specimens incubated with or without oxygen. The results indicated that the additional increase of glucose uptake with insulin stimulation was small. However, frogs are amphibians that live in water; they might be more resistant to hypoxia than mammals.

The findings in that glycogen was spatially distributed in muscle was made in 1985 [145], where workers hypothesised that this occurred in response to anoxia. However, investigations of the biochemical viability of incubated muscle revealed that the energy levels were unchanged during the procedure and it was concluded that the incubated muscle specimens were suitable for metabolic studies [141, 146]. In 1991, the size of tubular muscles preparations were considered [144], workers observed that smaller muscles from mice was preferable for *in vitro* analysis compared to rat preparations. In one study, comparison were made between rat *epitrochlearis* muscle specimens, which was validated earlier [156].

However, a positive control for the incubation *per se* has not been considered in earlier studies. Most controls used in the earlier incubation protocols is a muscle incubated under the same conditions, without including the perturbation under study (i.e. insulin or a metabolic stressor). In *paper 2* and *3*, a control for the incubation step was included. The result from these studies indicates that the incubation step *per se* influences the glycogen concentration of the isolated muscle, *paper 2*, as well as the insulin specific signalling events, *paper 3*.

4.2 THE SYSTEMS BIOLOGY APPROACH

The objective of this thesis has been to use a systems biology approach to investigate the effect of insulin signalling on glucose homeostasis within skeletal muscle.

However, previous research has only considered separate parts of the glucose metabolism system at one time. Hence, a holistic view that is thought to come with systems biology has not been considered. Even though, one can discuss whether a systems biologist has a more holistic view than an experimentalist within molecular biology or biomedicine [19].

The holistic view that is applied by the systems biologist requires data from the whole system to be addressed at once. The system can be the entire cell, with all its components interacting, or just part of it, which is more common [21, 75, 130]. The system can be studied from a bottom-up or top-down view (figure 2). The bottom-up view is the most frequent used approach [21]. However, if data is from an experimental system that uses the top-down approach, the systems biology approach would preferably be that as well, and *vice versa*. A bottom-up description of the system, such as the effect of insulin signalling on glucose metabolism, is presented earlier [87, 130]. These mathematical models use phenomenological functions to model the molecular interactions. One of the most frequent phenomenological functions is the Michaelis-Menten kinetic, which describes a sigmoid function. However, with this approach, one assumes that the substrates are in excess. A modification of this assumption is the Goldbeter-Koshland model [160], which describes a step-function. The Goldbeter-Koshland approach [27, 160] allows the substrates to be in the same order as the enzymes, which is most likely a more physiological situation.

The bottom-up approach always ends up with unknown parameters that cannot be either measured or estimated [21, 22]. Conversely, the top-down approach that was used in *paper 1* may not be suitable to answer questions regarding molecular interactions. To select the appropriate approach is not trivial. However, the process of developing a mathematical model *per se* will increase the understanding of the molecular mechanisms that works within the system, independent of the view of the system.

Even though that the top-down approach has been used before to elucidate the impact of the experimental design [127, 148], the assumptions made in the spatial model have never been addressed earlier, *paper 1*. The most important assumption made in the models [127, 148] and in the experimental handling of the tissues (i.e., the use of lysates from whole muscle preparation before measurement) assumes that the sample is homogenous. The muscle tissue is known to be heterogeneous, with different fibre types and specific fibre distributions [62, 64, 65, 161, 162]. This by itself points towards the importance of a spatial, compartmentalised experimental, as well as a mathematical model.

4.3 AVERAGING DATA

The necessity of having enough material to obtain accurate measurements has driven the experimental design towards homogenising the subject that is studied. This procedure can be defended if the entities under study are approximately homogeneous and synchronised, or if the entire population of cells are considered. However, with unsynchronised entities, e.g., signalling pathways or cells, the interpretation of data will be obstructed. In this thesis, data from both whole muscle preparations, as well as single fibres within the intact tissue has been used. In *paper 1*, the metabolic data was derived from whole muscle lysates. Hence, averaging of the performance of all single muscle fibres was made. The intention of using these data was to address and validate

the assumption made when data are collected and further processed before measuring. These assumptions are;

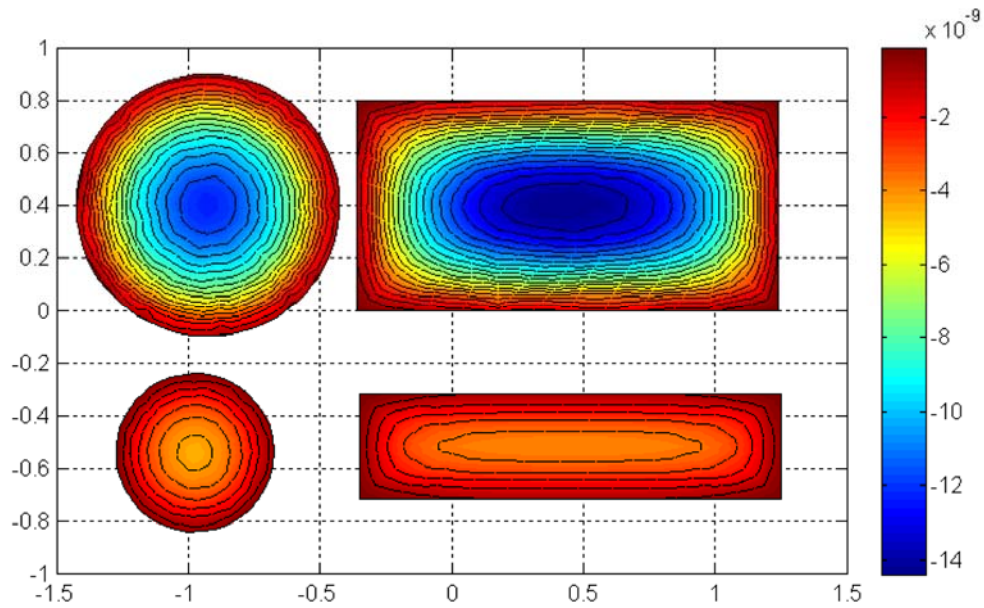
- Homogeneous tissue
- No affect of anoxia on glucose metabolism
- Steady-state or fixed end-point measurements

4.3.1 Homogeneous tissue

A skeletal muscle is heterogeneous in regard to its fibre type composition [59, 62-65], as well as its response regarding glucose metabolism and insulin signalling capacity [68], *paper 3*. However, the heterogeneous fibre structure that characterises skeletal muscle is handled by preferably using muscles with more homogenous fibre composition [141], such as the EDL and *soleus* muscle. These muscle types are used as models for different fibre types since the EDL is predominantly composed of glycolytic fibres and soleus of oxidative fibres.

4.3.2 Muscle geometry

The EDL and *soleus* muscles have tubular geometry. The rationale behind the muscle type used is dependent on the scientific purpose of the specific experiment, such that the *soleus* and EDL have distinct fibre type composition and each have a tendon that facilitates surgery. However, the tubular geometry might have an impact on the ability of oxygen to diffuse into the muscle [141]. Conversely, the *epitrochlearis* and the *diaphragm* muscle are assumed to be sufficiently thin to permit oxygen diffusion into all fibres [141]. The disadvantage of using tubular muscles compared with more flattened muscles such as the *epitrochlearis* or the *diaphragm* muscle is thought to be related to the limitation of oxygen diffusion. This condition has been circumvented by using smaller tubular muscle specimens [141, 144] from mice rather than rats. However, data indicating that diffusion is affected by the shape or the geometry of the muscle is lacking. A simple computational analyse of the diffusion and consumption of a component that exists outside the geometry indicates that the geometry *per se* has no effect (figure 12), instead the diffusion distance is important, as indicated by Hill and Barclay [127, 148] and Henriksen & Holloszy [144].



-Figure 12. Computational simulation of an optional component for diffusion and consumption in different geometries. The model used is $-D\Delta c = Q(c)$ with the parameters, D = diffusion constant = 5×10^{-5} , $Q(c)$ = consumption rate inside the geometry = 1×10^{-10} . And c is concentration of an optional component. The concentration field is shown, where red is higher concentration the blue. The two geometries in the bottom are half the size of corresponding upper panel. Simulation was done by using MATLAB, (MathWorks, www.mathworks.com).

4.4 ADVANTAGEOUS OF A FIRM TRANSLATIONAL DESCRIPTION BETWEEN SCIENTIFIC COMMUNITIES

A mathematical description is advantageous as it gives a firm representation of the phenomena under study. The mathematical model can then be tested and reiterated. The results from these tests can be used to adjust the model, even though this is not always suitable as the reformulation of the model is a new model with different characteristics, and the firm representation is then disrupted. To have a well defined description of the problem will pin-point all weakness in the hypotheses that describes the phenomenon. The benefit of having the phenomenon described with a mathematical model is best seen when a specific problem is copied with, for example *paper 1*.

To really get the most of this advantage, a common description of molecular network would be favourable for the understanding of the phenomenon [163, 164]. Today such a standard is lacking, even though, several proposals have been discussed [163-165]. One of the properties needed in a common firm description is a cartoon that could be unambiguous translated into a mathematical formulation. The cartoon used to describe the mathematical model in *paper 1*, and in figure 5 has this property. The main advantage of such a description of the molecular network, beside the ability to unambiguously translate it into a mathematical description, is that the number of symbols is small and, therefore, easily remembered (figure 5).

Cartoon models that are generally derived by experimentalists, see for example [166](), have the disadvantage in that consequences of a certain proposed function cannot always be strictly followed. This analysis can be done in the system outlined in figure 5 and in *paper 1*, as well as in the proposed standards. The proposed standards, however, have numerous symbols for different molecular events [163-165]

that might be hard to remember, especially if the model contains lot of different species and events. The main disadvantage with the system used in *paper 1*, and in figure 5 might be that it is not even close to the cartoon-system used in cartoons derived by both experimentalists, as well as the proposed standards [163-165], and could then feel unfamiliar. However, independent how the cartoon-models are drawn, it is important to adapt a system that makes explicit analyses possible, just by studying the interactions drawn.

4.5 MODEL DEVELOPMENT PROCESS

4.5.1 Formulation of hypotheses

The objective in *paper 1* was to evaluate which of the following hypotheses that explains the biochemical observation of high lactate production from the incubated muscle [145, 147, 152] (figure 13) and the lack of an increase in glycogen content after the incubation with insulin [145, 152]. The dominant hypotheses put forward to explain the high lactate production is that a mechanism in glycolysis work as a safety valve to allow for an extended amount of intra-cellular glucose to be converted into lactate by fermentation [152]. In the alternative hypothesis, it was assumed that the incubated muscle specimens are affected by anoxia and that this cause the high lactate production [145, 147], and the subsequent decrease in glycogen concentration.

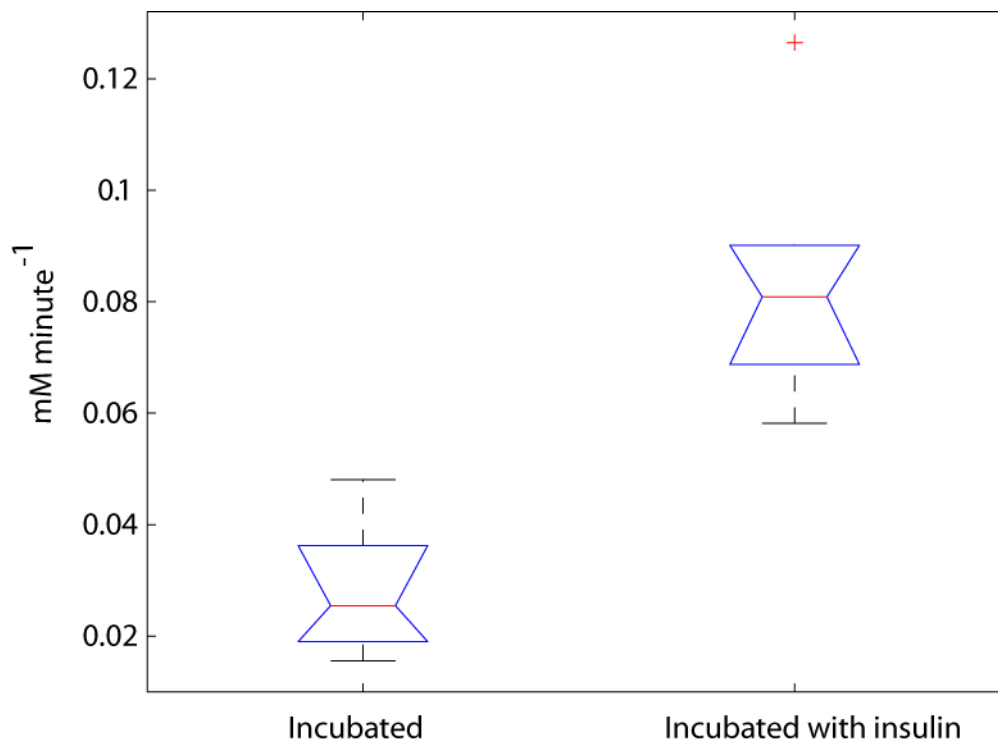


Figure 13. EDL muscle lactate production measured in the media after incubation, with or without insulin stimulation. A significant increase in lactate production was observed after insulin stimulation. The increase was hypothesised to be caused by either a safety valve mechanism or central anoxia.

4.5.2 Model complexity

The cause of the high lactate production was believed to be dependent on time, but not location within the muscle specimen. Therefore, ODE was used to describe the

molecular events considered, *paper 1*. The regulation of glucose uptake and utilisation by insulin are complex and highly regulated molecular processes [56, 95, 106, 130]. However, the time-resolution in data from the incubated muscles specimens was poor, due to the fact that the data sampling was only taken under steady-state conditions, after sixty minutes of incubation. Furthermore, the assumption that the muscle tissues are homogeneous is an over-simplification, since these tissues are extremely heterogeneous [59, 61, 62]. Muscle-fibre plasticity [107, 167] may be an important consideration, thereby adding more complexity to the experimental design. Despite this knowledge, the mathematical model was chosen to consider one compartment, as it is important to use the same scale on the mathematical description as used for the data sampling to address the hypotheses.

4.5.3 The choice of model-complexity affects the results

The use of muscle preparations is a considerable simplification of the *in vivo* situation, which in turn may be hard to study all aspects on. Conversely, the use of cultured skeletal muscle cells also has drawbacks, as they have minimal differentiation into mature muscle fibres [142, 143]. To study complex phenomenology, models are used with the knowledge that their inherent simplification might affect the results.

The use of ODE in mathematical modelling, e.g., the simplification that space is un-important clearly affects the results; however, this is still done. In the mathematical model derived in *paper 1*, a fairly simple model formulation was the end result. The starting point was a highly non-linear model with fifteen state-variables and more than fifty parameters. However, the model identification process resulted in the OCM presented in *paper 1*, with two state variables and five parameters.

4.5.4 Mathematical model identification process

To identify the mathematical model, parameters should be uniquely determined [22]. The model network was extracted from several publications of the effects of insulin on glucose uptake and utilization. However, the model was unidentifiable, as the available data only consists of five observations, before and after insulin stimulation, *paper 1*. To estimate the parameters in a more complex model, such as the model presented by [130], it is necessary to have access to more informative data [22, 132].

4.5.5 Parameter estimation

The estimation of model parameters in *paper 1* was straight forward, as the model identification reduced a complex bottom-up model to an identifiable top-down model. The steady state assumption was used and since almost all processes were measured, the determination of the parameter was simple. In the case where the molecular process was not measured, the glycogenolysis, two equations could be used to solve this parameter, *paper 1*. The numerical value of the parameter in this process differed, due to the fact that the system was not in steady state.

4.6 ANALYSES OF THE MATHEMATICAL MODEL

4.6.1 Steady-state

The steady state condition is favourable for the analysis of a mathematical model consisting of differential equations. By setting the right side of the equations to zero

and solving for the entity of interest, an expression is created. The steady state analyse of the mathematical model in *paper 1* revealed that only one of the equations, the variable *G6P*, was in steady state, thus, the system was in a quasi steady state. This result was expected, as the glycogen content measurement was done as an end-point measurement. When measuring end-points, the actual timing is crucial, as deviation affects the actual number. This is not the case when measuring processes that are at steady-state.

A set of two differential equations can be globally analysed by means of a phase portrait [24, 26]. In such analysis, the stability of the steady-state point can be visualised and analysed in a way that is easy to adapt. The concepts that are of interest are the existent of steady-state points, their number, and the qualitative characteristics. These qualities are determined by calculation. However, the characteristics of the steady-state point and the systems dynamics can also be visualised by using the phase portrait, (figure 14).

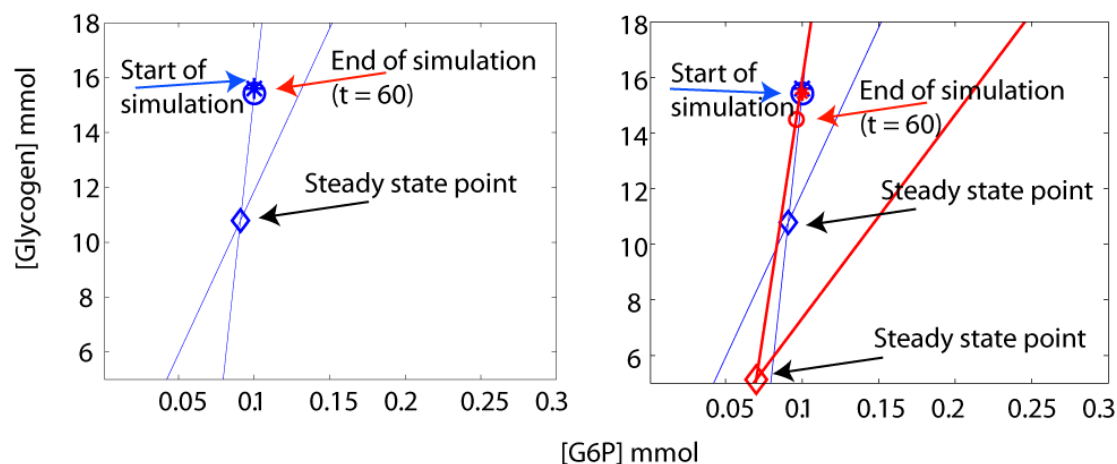


Figure 14. Phase portrait of the one compartment model. The one compartment model, *paper 1*, global behaviour is visualised in a phase portrait. Left panel describes the basal state, and the right panel describes the insulin stimulated state as shown by the red lines. Time (0) is marked with a star, blue for the incubation control state and red for the insulin-stimulated state. A circle marks the “end of simulation” and a diamond marks the steady-state point. The steady-state points are stable. Hence, the systems solutions are moving towards these coordinates. Note that the coordinates for the steady-states points are smaller than the points for the start and end of simulations, meaning that with an extended experimental or simulation time, greater glycogen depletion will occur. The parameter values are derived from table 1 in *paper 1*.

4.6.2 Parameter sensitivity

The uncertainty that exists in parameter estimation might affect the global dynamic of the system. To investigate the effect of small perturbations in the parameter values, a sensitivity test was performed [73, 74]. This analysis reveals which of the parameters that affect the steady-state conditions most, and therefore identifies the parameters that are interesting for follow-up studies or parameters that should be best estimated. The parameter sensitivity analysis performed for the mathematical model in *paper 1*, highlighted that the cause behind the high lactate production was best explained by the anoxia hypothesis.

4.7 REDUCED MATHEMATICAL MODEL

4.7.1 Quasi steady-state

The quasi steady-state of the mathematical model in *paper 1* was further used to reduce the set of two differential equations into a single differential equation that could be explicitly solved. The entities in the reduced model were all measured. The solution allowed for an estimation of the changes in the glycogen content after the muscle specimens were stimulated with insulin, a characteristics that was not assessable before. Furthermore, combining the reduced model with the quantitative data on spatial glycogen content together with an estimation of the grade of anoxia, calculation of spatial glucose uptake can be performed. This is done below, in 4.9.

4.7.2 Empirical cumulative data distribution function

In the parameterisation of the models in *paper 1*, the median value was used to estimate the different parameter values. This might affect the model outcome, as the different data-distributions were skew, *paper 1*. To analyse the model prediction without this drawback, empirical cumulative distribution functions (ECDF) were made of all data distributions, one for each of the measured process (figure 9). Linear interpolation was performed between the single data points in every ECDF and monotonic increasing continuous functions were created. One hundred simulations were performed where the data points used to calculate the parameter values was randomly drawn from the ECDF's. A statistical test on the result from the model was performed. In the basal state, it was a two-sided Student t-test, and in the insulin-stimulated state, it was a right-sided Student t-test. The results clearly indicate that in the insulin-stimulated state, glycogen levels will always decrease; in the basal state a tendency to decrease ($p = 0.06$) was observed, but no statistical significance was obtained, *paper 1*.

4.8 QUANTITATIVE ANALYSIS OF IMAGES OF TISSUE PREPARATION

To understand the regulation of glucose homeostasis in skeletal muscle, data with high resolution is required. Staining of cryosections from muscle preparations allows for a series of images to be taken. The intensity of the staining can then be analysed by computational methods, i.e., image analysis, *paper 2* and *paper 3*. The use of dyes or immunohistochemistry based techniques has certain limitations that restrict their use for quantitative analyses. Staining with dyes, either directly or by enzyme-mediated immunohistochemistry can be made quantitative by using a standard curve. The function that describes the standard curve will then be used to transform the intensity of the values from the sample images to concentration values (figure 10). However, the use of immunofluorescence has been optimised to give maximal amplification of the signal. The drawback with this technique is to quantify the light emitted from fluorophores bound to the antibody, knowledge of the relationship between (1) the primary antibody and the molecular target, (2) the primary and secondary antibody, (3) the amount of fluorophores bound to the secondary antibody, is required (figure 8). The method to calibrate results between the standard curve and the signal will approximately estimate the concentration, if the ratio of noise to signal not is too high. In images from cryosections of a tissue, based on immunofluorescence, the possibility exists to identify single particles as, done when analysing single-molecular movements in cell-cultures [136, 138]. The technique of image analysis and processing can create a

3-dimensional high resolution picture of the entire tissue [168]. The understanding of glucose homeostasis would probably benefit of such an analysis.

4.8.1 Periodic acid Shiff staining and quantitative imaging

Quantification in the meaning of transforming data from a standard-curve is a standard procedure. To get a good estimation of the function describing the standard-curve, repeated measurements of new standard samples are required. The repeated measurements of the same data will allow for an estimate, not only of the variance introduced by the equipment use, but also of the biological variance. The standard-curve is not required to be linear. The sample-measurement can be transformed by the standard-curve-function into concentration.

PAS staining gives a magenta colour when reacting with carbohydrates. The magenta colour consists of red and blue, which in turn is part of the RGB colour space. To analyse the amount of magenta staining in muscle tissues, the intensity of the green colour was used. The greyscale has previously been used to transform glycogen as measured by PAS staining into concentration [140]. However, the greyscale depends on the specific software used, since the transformation from the RGB colour model into the greyscale depends on the weight used in the transformation formula. In contrast, the green channel is, well defined.

4.8.1.1 Sorting data and group size

The cryosections from tubular muscle specimens is circular. The best representation of a section of a circle is a circle sector. However, computationally it is easier to work with a rectangular section, even though there will be an over representation of objects in the centre. To avoid such over representation, a small rectangle may be used (figure 11). The use of a rectangle will have an increased influence on the background at the border of the segment (figure 11). This is corrected by sorting all rows in the matrix that make up the images, *paper 2*, and *paper 3*. The sorting algorithm has a threshold value that is close to the intensity of the border. The sorted matrix will not have any background signal at the border; however, it may be skewed (figure 11). Since the skewed matrix will be analysed, manual inspection is required. To get a good estimate of the signal-gradient within the stained tissue, several cryosections should be used. In the image analysis, *paper 2* and *paper 3*, three sections were applied for each image (figure 11). The mean value of these three sections was used for further analysis. The size of each pixel-group was assigned to cover two to three cells, (figure 11).

4.8.1.2 Normalization of spatial data

The aim of analysing the spatial distribution of the glycogen concentration, *paper 2*, or insulin signalling, *paper 3*, was to determine whether deviations from a uniform distribution could be observed. The normalization of data to the pixel group that represents the superficial cells makes it possible to address this question (figure 11), *paper 2* and *paper 3*. It was expected that in a homogeneous sample, the curve created after normalisation would be straight, without any slope. An increasing slope indicates that adjacent fibres towards the centre of the tissue would have a mechanism that can increase glycogen content, *paper 2* or insulin signalling, *paper 3*. Conversely, a decreasing slope would indicate that these cells have a mechanism that decreases the

glycogen content, or insulin signalling. The glycogen content, *paper 2*, significantly decreased in the core of the incubated muscle specimens. The molecular mechanisms responsible for this are either an increase in glycogen consumption or a lack of glucose uptake, which also would increase glycogen breakdown. Both mechanisms will be driven by the need for each cell to preserve energy homeostasis (figure 5).

4.9 MOLECULAR MECHANISM INVOLVED IN ENERGY HOMEOSTASIS DURING *IN VITRO* INCUBATION OF SKELETAL MUSCLE

The mechanism explaining the decrease in glycogen content and the pattern observed in earlier studies [145, 147], as well as *paper 2*, is speculated to be accounted for by one or a combination of the following;

1. anoxia in the core of the incubated muscle specimen
2. insufficient insulin action in the core of the incubated muscle specimen
3. insufficient glucose concentration in the core of the incubated muscle specimen

4.9.1 Hypotheses testing

Two current hypotheses exist to explain the data regarding the unexpected high lactate production and lack of an increase in glycogen content during the muscle incubation. In *paper 1*, we used the derived mathematical models to analyse these hypotheses. The “glycolysis spill-over” hypothesis explains that high lactate production will work as a safety valve mechanism in the glycolytic pathway [152]. The availability of glucose is assumed to be so high that the muscle fibres cannot fully metabolise the available substrate, and to avoid dangerous intra-cellular concentration of glucose; the excess is fermented and re-exported to the media as lactate. This mechanism is enhanced when the muscle is stimulated with a super-physiological concentration of insulin. The experimental data that supports this hypothesis originates from studies on transgenic animals [153, 154], where either the amount of glucose transporter 1 [154] or 4 [153], have been increased. Lactate production is increased in both of these animal models.

Given that the one-compartment-model *paper 1*, is identified, certain analyses can be performed to try to explain the available observations with the model. Either is the system, both the biological and the mathematical, in steady state; which is to state that no further increase in glycogen content can be expected, was in fact unexpectedly observed in data from whole muscle preparations. Or is the system in quasi steady state, and if this is the case, the glycogen concentration would increase, which was not observed in data from whole muscle preparations, but in the spatial measurements of glycogen, *paper 2*. The steady state analysis of the model supports this latter analysis, *paper 1*. The sensitivity analysis performed with the two parameters, representing lactate production and glucose oxidation, *paper 1*, varied in an extended data-frame, with no available solutions obtainable to support the glycolysis spill-over hypothesis.

The hypothesis on central anoxia as the mechanism for the high lactate production and the lack of an increase in glycogen concentration cannot be explained by the biochemical data in *paper 1* without adding more information. The procedure used to challenge this hypothesis was to assume that the total amount of energy produced from oxidation and fermentation of glucose derivatives is a property that is constant for a specific situation. Calculations of the amount of energy extracted from either oxidation or fermentation of glucose derivatives was sum up to get this constant, *paper 1*. Assuming that no energy was produced by fermentation of glucose

derivatives, i.e., no anoxia present, instead this amount of energy was produced by oxidative phosphorylation of glucose derivatives. The transformed data was used to estimate new parameter values. The results gave that there was a possible solution of the system. Hence, the analysis of the model supports the hypothesis that anoxia is the cause behind the high lactate production by the incubated muscle specimen.

4.9.2 Anoxia

The intra-cellular energy supply can theoretically be produced by mainly two different mechanisms; oxidation of either amino acids, lipids, glucose derivatives, or by fermentation of a glucose derivative (figure 5). Oxidation requires oxygen in sufficient amount. In vivo, oxygen is transported from the lungs by the circulation bound to haemoglobin. The blood-vessels are divided into capillaries that keep a constant blood volume in muscle to enable the diffusion of oxygen into each muscle fibre [123]. The oxygen is stored bound to myoglobin [169]. Red muscle fibres have a high content of myoglobin [169]; these muscles also have a high content of oxidative fibres [170] and more capillaries [125]. *Soleus* is a red oxidative muscle, and EDL is a white glycolytic muscle. In the *in vitro* incubated muscle specimen, the circulation and the regulation of the capillary network is interchanged with oxygen diffusion from the muscle specimens from the media.

The assumption of a fully oxygenated muscle specimen during the incubation procedure is supported by several earlier studies [79, 127, 141, 148]. The earliest report are almost a century, where a mathematical model of frog *sartorius* muscle incubated under similar conditions was derived [127]. A minimal diffusion distance was defined by Hill [127], (figure 7). The aim of that study was to investigate whether anoxia was a confounding factor that should be accounted for. The minimal diffusion distance that was defined was above the radius of the muscle specimens under study. Hence, it was concluded that muscle specimens below that size were suitable for *in vitro* incubations. The assumptions that were stated in the description of the model, considered a homogeneous structure and a critical value of oxygen pressure to be above zero. In 2005, the model developed by Hill [127] was further analysed for the mouse EDL and *soleus* muscle after *in vitro* incubation [148]. The results obtained indicated that the oxygen pressure was above zero in the core of the tubular EDL and *soleus* mouse muscles. However, if the muscle specimens underwent contraction during the *in vitro* conditions, the critical diffusion distance decreased to the superficial fibres [148].

The frog *sartorius* [79] and *diaphragm* [171] muscles were used to study the effect of anaerobiosis on glucose uptake. The incubation media was gassed with either 95% O₂, 5% CO₂ or 95% N₂, 5%O₂. The muscles incubated in the presence of a nitrogen gas were defined to be incubated under anoxic conditions, whereas muscle specimens incubated 95% O₂ were defined to be incubated under oxygenated conditions [79]. These definitions have been used subsequently by contemporary workers [141, 144, 155, 172-174].

A spatial analysis is required to analyse whether or not the muscle specimens actually sense low oxygen levels. One way to determine this is by the use of immunofluorescence with antibodies detecting HIF1-alpha, *paper 2*. If the results indicate an increased content HIF1-alpha in the core of the incubated muscle specimen, then the samples are sensing a low oxygen level. The sense of low oxygen levels requires that fermentation of glucose derivatives occurs to maintain energy homeostasis. Since fermentation is an inefficient way of producing energy [175], *paper*

1, the demands of available intra-cellular glucose will increase. If these demands can be met without breaking down glycogen, then the anoxic milieu might not affect the interpretation of insulin's response on glucose metabolism. However, if the demands cannot be met by an increase in glucose uptake, anoxia will become a confounding factor [150, 151] that will obstruct the understanding of glucose homeostasis within this experimental setting. In *paper 2*, staining for HIF1-alpha was increased in the core of both the oxidative soleus muscle and the glycolytic EDL muscles. This increase in staining was super-imposable with the area observed to have decreased glycogen concentration. These results indicate that glucose uptake is insufficient in the core of the incubated EDL and soleus muscle, *paper 2*.

4.9.2.1 Apoptosis

A question can be raised regarding the severity of the glycogen depletion in the incubated muscle and whether it may affect the viability of the muscle fibres. The observation that a core forms due to glycogen depletion has been known for almost three decades [145, 147]. The authors put forward the hypothesis that the core was formed due to anoxia, causing the glycogen depletion [145, 147]. However, studies on the energy levels have shown that they are unaffected [78, 79], and the conclusion was that the muscle specimens are biochemical viable [146].

To avoid necrosis, apoptosis is a molecular mechanism that allows cells to be degraded in an ordered way [176]. Apoptosis is a molecular pathway that has several check points before it is irrevocable [177]. The last step is the cleavage of caspase-3 into two subunits [178]. One circumstance that triggers the apoptotic pathway is low energy storages levels [179]. In *paper 2*, immunofluorescence studies were performed to determine whether the active form of caspase-3 was present in the incubated muscle. The results provide evidence to suggest that the oxidative *soleus* muscle was slightly protected against apoptosis, compared to the glycolytic EDL muscle. *Soleus* muscle has an enhanced insulin signalling capacity compared to EDL muscle [68], *paper 3*. This possibility to increase the glucose uptake might be a factor that partially protects insulin stimulated soleus muscles from apoptosis, *paper 2* and *paper 3*.

4.9.3 Insulin concentration is sufficient to trigger its signalling in the core of incubated muscle specimen

The hypothesis that insulin signalling was compromised due to insufficient insulin diffusion into the core of the muscle was investigated in *paper 3*. The rationale for the hypothesis is that if insulin insufficiently triggers its signalling cascade, GLUT4 would be inadequately transported to the plasma membrane, due to the hysteresis effect [87]. Hence, the amount of glucose transported to the intra-cellular compartment would be unaffected. The lack of available intra-cellular glucose and the anoxia, *paper 2*, would cause glycogen to be metabolised due to energy demands that could not be met by oxidation. However, the results provided evidence that insulin diffused into the core in sufficient amount to trigger its downstream signalling cascade, *paper 3*.

4.10 THE REDUCED MODEL

The reduced mathematical model developed in *paper 1*, can be used to estimate the total glycogen content after insulin stimulation. The requirement is that glucose uptake and utilisation as energy production is measured and that the glycogen content before incubation is measured.

$$GLY_{t(end)} = GLY_{t(0)} + \alpha t$$

Where the parameter α represents the difference between glucose uptake and utilisation as energy production and GLY_i is the time point for glycogen measurement, $i=[0, \dots, t(end)]$, t = time.

The combination of data obtained in *paper 2* and the mathematical model presented in *paper 1*, insulin's effect on glycogen concentration was predicted.

$$GLY_{t(end)} = 0,18 + 0,049 \cdot 10^{-3} \cdot 50 = 0,182 \text{ Molar}$$

The calculated increase in glycogen content after fifty minutes of insulin stimulation in the EDL muscle that is estimated is under the range of what was measured in the superficial fibres in *paper2*, (~0,25 Molar). The discrimination between the estimated values with the reduced model and the measured is incompletely understood. However, the constant α is derived from data obtained from whole muscle lysates, *paper 1*, whereas the glycogen concentration is from high resolution data, *paper 2*. The expansion of the solution to the reduced model, *paper 1*, might give an explanation.

$$\frac{dGLY}{dt} = k_1 - G6P(k_2 + k_3)$$

The α -value (0.049 mM/minute) is calculated assuming that no anoxia was present, *paper 1*. That is to say that the area considered is the same as that covered by group one to three when determine glycogen concentration (figure 11), *paper 2*, for EDL muscle after insulin stimulation. The only parameter that is still obtained from whole muscle preparations is the estimation of glucose uptake, *paper 1*.

$$k_1 = \frac{GLY_{t(end)} - GLY_{t(0)}}{t_{(end)}} - G6P(k_2 + k_3)$$

The estimated glucose uptake is 0.14 mM / minute. It is approximately two-fold greater than the value measured, if the median value is considered, *paper 1*. However, it is in the upper range of measured data for glucose uptake, *paper 1*. It might be that the diffusion – consumption of glucose play a role (figure 12).

5 SUMMARY

In this thesis, mathematical modelling and immunohistochemistry has been used to validate data on insulin-stimulated glucose metabolism obtained from mouse muscle preparations. Specifically, two previously stated contradicting hypotheses on whether the observed lactate production was due to anoxia or a safety valve mechanism in glycolysis has been tested. The hypothesised limitation on measuring glycogen content in whole muscle was shown to be due to averaging unequally distributed data. Lactate production and the unequally distributed glycogen data was due to anoxia and not due to insufficient insulin action in the core of the muscle preparation. The derived mathematical model can be used to estimate glycogen concentration in whole muscle preparations, and to estimate the fibre specific glucose uptake rate. Insulin signalling was confirmed to be higher in oxidative fibres compared to glycolytic fibres in the same specimen i.e. the same environment.

- The *in vitro* incubation of skeletal muscle *per se* influences the production of lactate and subsequently decreases the glycogen concentration in the central portion of the specimen.
- The rate of high lactate production is best explained by the anoxia hypothesis.
- The central depletion of glycogen is severe enough to induce apoptosis.
- Insulin diffuses in sufficient amount to trigger its signalling cascade throughout the entire muscle preparation.
- Insulin signalling is higher in oxidative fibres, compared to adjacent glycolytic fibres.
- Insulin stimulation of soleus muscle increases the central glycogen concentration compared with the incubated positive control, but not compared with the negative control.
- Mathematical models are derived, such that it is possible to estimate the glycogen content and glucose uptake, without confounding anoxia effects.

6 CONCLUSION AND FURTHER PERSPECTIVES

The aim of this thesis was to use a holistic view of systems biology to add new knowledge on insulin-stimulated glucose metabolism in skeletal muscle. Mathematical models are a powerful tool to describe complex molecular events. Depending on the problem stated and the information available, different types of mathematical models are used. The most common approach is to describe networks of molecular interaction using a set of ordinary differential equations [24, 25]. The mathematical model presented in *paper 1*, is a set of two ordinary differential equations. The quasi-steady-state that the system of differential equation was in made it possible to further reduce the mathematical model into one differential equation, *paper 1*. The mathematical models facilitate the understanding of insulin action on glucose metabolism in skeletal muscle *in vitro*. The top-down approach and the analyses of the model revealed that anoxia is most likely the cause of both the previously observed high lactate production and the lack of an increase in glycogen content under insulin-stimulated conditions. The predictions from the model highlighted a need for higher resolution experiments to show whether glycogen concentration was affected by anoxia or not. The histochemical studies combined with protein specific immunofluorescence staining allowed for analysis of single fibres within a tissue.

A novel hypothesis considered in this thesis dealt with the concept of deficient insulin signalling, coupled with a lack of glucose uptake and glycogenesis as the cause of the central glycogen depletion in the incubated muscle. The rationale for this hypothesis was that if the expected increase in glycogen concentration was missing, it might be that insulin may not have diffused into the core of the incubated muscle preparation. Hence, no increase in glycogen concentration was possible. This effect could be an additional effect over and above the development of central anoxia.

In *paper 2*, the prediction made in *paper 1*, regarding the presence of anoxia as the cause behind the lack of increase in glycogen content was tested and verified. In *paper 3*, the hypothesis on deficient insulin signalling was falsified and hence, central anoxia is most likely the cause behind the observed increase in lactate production and the central breakdown of glycogen. Even though it cannot be ruled out that low central concentration of glucose might be at hand [150, 151].

The use of systems biology approach in this thesis increased the understanding of the effect of oxygen on glucose metabolism in the incubated muscle preparation. This is a problem initially stated a century ago by Hill [127], where the minimal diffusion distance and the critical intra-cellular oxygen pressure, was established. These concepts by Hill have been virtually unchallenged. One of the main assumptions in Hill's work [127] was that the skeletal muscle was a homogenous organ. Today it is clear that skeletal muscles are composed of several types of fibres [59, 61, 62]. Each fibre type has a distinct characteristic regarding its oxygen consumption profile [180]. Moreover, the more oxidative fibres are distributed central in the muscle compared to the less oxidative fibres [62-65], hence, skeletal muscle is a heterogeneous tissue and the simplification made when measurements are performed on lysates from whole muscle disregards this fact. The other main assumption was that the critical value of intra-cellular oxygen pressure is zero. This assumption has still to be challenged.

To further increase the knowledge on insulin action on glucose metabolism in skeletal muscle, data on single-cells would be beneficial. To achieve this, immunohistochemical methods are preferred. To get the most out of the data, these methods should be as quantitative as possible.

With histochemical and enzymatic immunohistochemical methods, standard-curves are available to get acceptable quantitative data. With immunofluorescence based methods, more effort has to be made to achieve analytical methods that in combination with a standard-curve, or not can extract data on the level of concentration at least. However, as immunofluorescence allows for detections of single molecules [136, 138], the aim has to be the detection of those entities. Image acquisition techniques that are available today allows for detections of approximately 40 frames per second to be stored and handled by the software. Furthermore, computational techniques make it possible to take the images and get a 3-dimensional-structure that can be further analysed [168].

To get a better understanding of skeletal muscle glucose homeostasis an experimental procedure that avoids the confounding of anoxia would be beneficial to use. Especially, when the experiments are used to increase the understanding of the development of peripheral insulin resistance in complex diseases such as T2DM, see for example [181-184]. One way to avoid anoxia is to perform the experiments on whole animals. An intraperitoneal injection in combination with a measurement of both blood and lymphatic glucose and insulin levels may make it possible to investigate the effects on single fibres after dissecting the muscle. The advantage of such a procedure is that confounding effects of anoxia can be avoided, and that knowledge of the amount of both glucose and insulin can be monitored, before and after it affects the muscle.

Homeostatic regulation is a system property that best is understood by a top-down approach. The top-down approach does not have to disregard information on single components and their interactions, instead it is important to understand the single small systems behaviour in isolation, and then combine those motifs into a comprehensible model, mathematical, verbal as well as graphical representation. The question raised to the models should preferably be driven by specific hypothesis.

By combining the methods mentioned, the possibility to understand the basal and insulin action on glucose metabolism can grow. Hence, our understanding of the complex nature of the regulatory networks that cause dys-regulation of glucose and energy homeostasis can increase.

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We all know that the combination of different scientific fields with their diverse traditions is often very fruitful but demands much time, deep considerations, open minds and extra efforts of all involved. I do believe that we all have learned something of the process of combining our disparate disciplines and I hope that we all have something positive to take along into the future. Thanks again all of you for the support and for joining me in this project.

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The scientific milieu that I have been in the last eight years, before and during this project is a combination of four research groups. I started to be a member of the Computational Biology Research Group at University of Skövde. Friends, we had a lot of fun and it was especially interesting to get an insight into so disparate scientific questions such as the cause of the shape of bio films to the spread of pneumococcal among children at kinder gardens. And you, we still miss some celebrations!

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“de ska va got å leva”

Galenskaparna och After Shave

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