INSULIN SIGNALING DYNAMICS
IN HUMAN ADIPOCYTES

MATHEMATICAL MODELING REVEALS MECHANISMS OF
INSULIN RESISTANCE IN TYPE 2 DIABETES

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Linköping 2014
During the course of the research underlying this thesis, Elin Nyman was enrolled in Forum Scientium, a multidisciplinary doctoral program at Linköping University, Sweden.
It always seems impossible until it’s done

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ABSTRACT

Type 2 diabetes is characterized by raised blood glucose levels caused by an insufficient insulin control of glucose homeostasis. This lack of control is expressed both through insufficient release of insulin by the pancreatic beta-cells, and through insulin resistance in the insulin-responding tissues. We find insulin resistance of the adipose tissue particularly interesting since it appears to influence other insulin-responding tissues, such as muscle and liver, to also become insulin resistant.

The insulin signaling network is highly complex with cross-interacting intermediaries, positive and negative feedbacks, etc. To facilitate the mechanistic understanding of this network, we obtain dynamic, information-rich data and use model-based analysis as a tool to formally test different hypotheses that arise from the experimental observations. With dynamic mathematical models, we are able to combine knowledge and experimental data into mechanistic hypotheses, and draw conclusions such as rejection of hypotheses and prediction of outcomes of new experiments.

We aim for an increased understanding of adipocyte insulin signaling and the underlying mechanisms of the insulin resistance that we observe in adipocytes from subjects diagnosed with type 2 diabetes. We also aim for a complete picture of the insulin signaling network in primary human adipocytes from normal and diabetic subjects with a link to relevant clinical parameters: plasma glucose and insulin. Such a complete picture of insulin signaling has not been presented before. Not for adipocytes and not for other types of cells.

In this thesis, I present the development of the first comprehensive insulin signaling model that can simulate both normal and diabetic data from adipocytes – and that is linked to a whole-body glucose-insulin model. In the linking process we conclude that at least two glucose uptake parameters differ between the in vivo and in vitro conditions (Paper I). We also perform a model analysis of the early insulin signaling dynamics in rat adipocytes and conclude that internalization is important for an apparent reversed order of phosphorylation seen in these cells (Paper II). In the development of the first version of the comprehensive insulin signaling model, we introduce a key parameter for the diabetic state – an attenuated feedback (Paper III). We finally continue to build on the comprehensive model and include signaling to nuclear transcription via ERK and report substantial crosstalk in the insulin signaling network (Paper IV).

Vi studerar insulinresistens i fettceller eftersom fetma och typ 2-diabetes är starkt sammankopplade. Studier pekar även på att fettceller är de celler som tidigast under sjukdomsförloppet drabbas av insulinresistens. Fettceller från diabetiker skiljer sig från fettceller från friska när vi behandlar dem med insulin och mäter aktiviteten hos de proteiner som sänder insulinsignalen vidare genom cellen. Vi kan använda insamlade data från flera proteiner från både friska och diabetiker samtidigt och på så sätt hitta olika möjliga platser i cellen där insulinresistensen uppställt. Eftersom det är svårt att kombinera stora mängder data använder vi program som kan simulera data med hjälp av matematiska modeller och beräkningsalgoritmer. Med dessa hjälpmedel kan vi testa olika hypoteser och komma fram till nya experiment som skulle vara värdefulla för att komma närmare vårt mål – att förklara hur insulinresistens uppstår i fettceller.

Vi har alltså skapat matematiska modeller för utvalda proteiner som aktiveras av insulin. Genom att kombinera dessa modeller med data från friska och diabetiker har vi funnit en möjlig förklaring till hur insulinresistens uppstår i fettceller. Förklaringen bygger på en nedreglering av en i friska celler förstärkande återkoppling. Denna återkoppling påverkar de flesta proteiner eftersom den ligger direkt nedströms om insulinreceptorn – som insulin binder till på utsidan av cellen. Nedregleringen gör alltså att de flesta proteiner som normalt svarar på insulin får ett lägre svar och på så sätt sprids insulinresistensen i hela cellen. Tack vare användandet av matematiska modeller kunder vi med data av hög kvalitet från...
mänskliga celler, kan vi presentera denna unika systemförståelse av insulinresistens i typ-2 diabetes.

För att våra resultat också ska få en klinisk betydelse har vi kopplat samman våra matematiska modeller för insulinsignalering inom fettceller med modeller som beskriver blodsockernivå och insulinutsöndring för alla kroppens organ. För att kunna göra detta har vi formulat krav så att insulinsvaret på fettcellsnivå ska motsvara insulinsvaret på fettvävnadsnivå. Dessa krav kunde vi inte uppfylla direkt med en enkel sammankoppling av modellerna. Men med hänsyn till skillnader i exempelvis blodflöde och cellhantering (som kan verka stressande) kan modellerna simuleras tillsammans för att observera hur fetten interagerar med andra organ och vävnader. Sammankopplingen underlättar också studier av hur läkemedelsverkan sprids från celler där de har sin effekt, till resten av kroppens organ.


LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, referred to in the text by their roman numerals.

I  **Elin Nyman**, Cecilia Brännmark, Robert Palmér, Jan Brugård, Fredrik H Nyström, Peter Strålfors, and Gunnar Cedersund
*An hierarchical whole body modeling approach elucidates the link between in vitro insulin signaling and in vivo glucose homeostasis*

II  **Elin Nyman**, Siri Fagerholm, David Jullesson, Peter Strålfors, and Gunnar Cedersund
*Mechanistic explanations for counter-intuitive phosphorylation dynamics of the insulin receptor and IRS1 in response to insulin in murine adipocytes*

III Cecilia Brännmark*, **Elin Nyman**, Siri Fagerholm, Linnéa Bergenholm, Eva-Maria Ekstrand, Gunnar Cedersund, and Peter Strålfors
*Insulin signaling in type 2 diabetes – experimental and modeling analyses reveal mechanisms of insulin resistance in human adipocytes*

IV  **Elin Nyman***, Meenu Rajan*, Siri Fagerholm, Cecilia Brännmark, Gunnar Cedersund, and Peter Strålfors
*The insulin-signaling network in human adipocytes, normally and in diabetes – role of signaling through ERK1/2*
Submitted

* Authors contributed equally to the work
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AS160</td>
<td>Akt substrate of 160 kDa</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>Elk1</td>
<td>ets-like gene 1</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular regulated kinase</td>
</tr>
<tr>
<td>FOXO</td>
<td>forkhead class O transcription factors</td>
</tr>
<tr>
<td>GLUT1</td>
<td>glucose transporter 1</td>
</tr>
<tr>
<td>GLUT4</td>
<td>glucose transporter 4</td>
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<tr>
<td>IGF-1</td>
<td>insulin-like growth factor-1</td>
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<td>IR</td>
<td>insulin receptor</td>
</tr>
<tr>
<td>IRS1</td>
<td>insulin receptor substrate-1</td>
</tr>
<tr>
<td>MEK</td>
<td>mitogen activated ERK kinases</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>mTORC1</td>
<td>mammalian target of rapamycin, complex 1</td>
</tr>
<tr>
<td>mTORC2</td>
<td>mammalian target of rapamycin, complex 2</td>
</tr>
<tr>
<td>ODE</td>
<td>ordinary differential equation</td>
</tr>
<tr>
<td>p90-RSK</td>
<td>p90 ribosomal S6 kinase</td>
</tr>
<tr>
<td>PDK1</td>
<td>phosphoinositide-dependent kinase-1</td>
</tr>
<tr>
<td>PIP3</td>
<td>phosphatidylinositol (3,4,5)-trisphosphate</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PPARγ</td>
<td>peroxisome proliferator-activated receptor γ</td>
</tr>
<tr>
<td>S6</td>
<td>ribosomal protein S6</td>
</tr>
<tr>
<td>S6K</td>
<td>p70 ribosomal S6 kinase</td>
</tr>
<tr>
<td>sem</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>T2D</td>
<td>type 2 diabetes</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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<td>Tyr</td>
<td>tyrosine</td>
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INTRODUCTION

Diabetes is increasing worldwide with epidemic proportions. In developed countries, the prevalence of diabetes is 8-10% in adults, and out of these almost 90% have type 2 diabetes [1]. Type 2 diabetes (T2D) is a metabolic disease characterized by dysfunctions in the insulin control of energy homeostasis; both through a non-sufficient release of insulin, and an insulin resistance in the insulin-responding tissues [2]. The insulin resistance is initially compensated for by elevated release of insulin. When the increased release of insulin no longer can compensate for the insulin resistance in the target tissues, T2D can be diagnosed. T2D and the long-term complications of the disease cause individual suffering and are costly for the society since there is a lack of good treatments available. A main reason for the lack of treatments is that the underlying intracellular mechanisms to the disease are not fully understood.

We focus on the intracellular mechanisms behind insulin resistance in human adipocytes – the main cell population in the adipose (i.e. fat) tissue [3]. The insulin resistance in the adipose tissue results from an expanding storage of fat and is of particular interest, since it appears to influence other insulin-responding tissues, such as muscle and liver, to also become insulin resistant [2, 4]. The adipose tissue is thus one of the possible origins of T2D. The main task of the adipose tissue is to provide a reversible energy reservoir and thus to buffer for changing fluxes of lipids [5]. The adipocytes in the adipose tissue store lipids as triglycerides in a large lipid droplet that fills more than 95% of the cell volume, and the nucleus is squeezed into the remaining volume (Figure 1). Adipocytes also function as an endocrine organ and produce and secrete proteins (adipokines) as signals to communicate with other cells/organs [6]. We study the intracellular insulin response in adipocytes obtained from the subcutaneous adipose tissue of normal subjects and patients diagnosed with T2D, in order to unravel the mechanistic origin of insulin resistance.

As we understand more about signaling networks like the insulin signaling network, we progressively realize their inborn complexity. The further we reach in the research of intracellular signaling mechanisms, the more signaling intermediaries, crosstalk, and feedbacks are discovered [7]. To add on to this complexity, different kinds of ligands, as well as ligand abundance, location, and dynamics give rise to different intracellular responses [8]. To handle all this complexity and dissect the information in experimental
data, mathematical modeling is coming of age as an important tool for data analysis [9]. We use mathematical modeling as a tool to get maximal information out of our obtained experimental data and also to more intelligently design new experiments.

Figure 1: The morphology of an adipocyte
Adipocytes store triglycerides in a large lipid droplet that covers most of the cytosolic volume. The adipocyte nucleus is squeezed towards the plasma membrane.

It is important to increase knowledge of insulin resistance and T2D at the intracellular level, but perhaps even more important is to increase our knowledge of how these intracellular signals interplay with signals from other cells and organs. T2D is indeed manifested at the whole-body systemic level, and to relate our knowledge of insulin signaling in primary adipocytes with knowledge of the in vivo situation, we have developed a method to combine our intracellular models with whole-body models of glucose homeostasis. With such combined models, intracellular changes can be translated to whole-body responses, which is useful to simulate the effect of different treatments.

My overall aim with this thesis is to increase the knowledge of the dynamics of the intracellular signaling events in adipocytes in response to insulin by using mathematical models as a tool for data analysis. More specifically, I aim for a systems understanding of insulin signaling and insulin resistance, based on internally consistent data from human cells. In order to obtain such a systems understanding, we have integrated the modeling in the experimental work such that continuously modeling depends on experimental data, and
experimental data depends on modeling. I also aim for a proper link between intracellular human data and whole-body clinical data. In Paper I, we create such a link between mathematical models based on intracellular insulin signaling data on one hand, and mathematical models describing the dynamic flows of insulin and glucose on the whole-body level on the other hand. We discover the requirement for additional mechanisms to be able to link the *in vitro* and *in vivo* mathematical models and simulate *in vitro* and *in vivo* data simultaneously. In Paper II, we study the early dynamic insulin response in rat adipocytes and compare with the situation in human adipocytes. We learn that insulin receptor internalization is important for the dynamic behavior of phosphorylation and we find different explanations to an apparent reversed order of peaks in the phosphorylation dynamics. In Paper III, we study the whole network of insulin signaling for metabolic control – from insulin binding to glucose uptake and to control of protein synthesis – in adipocytes from normal and T2D subjects. With data from all the different signaling intermediaries we obtain a new systems understanding of insulin resistance. We build on these results in Paper IV, where we include the insulin control of the ERK signaling branch into our comprehensive mathematical model of insulin signaling and T2D. We use a minimal modeling approach to understand ERK phosphorylation dynamics. We also study crosstalk between insulin signaling in control of ERK and insulin signaling for metabolic control.

I have limited the scope of this thesis to mathematical models of insulin signaling in target cells, such as adipocytes and muscle cells. There are several efforts in modeling other aspects of T2D, for example with focus on beta-cell function and clinical disease progression. For a complete understanding of T2D, the different pieces to the puzzle must be combined, and such a combination benefit from the use of mathematical models. For the interested reader, modeling different aspects of T2D is reviewed in [10].
Mathematical modeling of intracellular signaling

Intracellular signaling systems are not linear pathways, but complex networks with positive and negative feedback loops and cross-interacting intermediaries [11]. The complexity of signaling networks aggravates the interpretation and understanding of experimental observations [12]. We therefore use mathematical modeling as a tool in data analysis, to be able to more formally test different hypotheses (Figure 2).

Figure 2: The modeling approach used in this thesis
Mechanistic hypotheses, i.e. mathematical models, are used together with experimental data to draw conclusions in the form of rejections and core predications. Model parameters are estimated and if model simulations do not agree with data for any parameter set, the model is rejected and we start over with new mechanistic hypotheses. If instead there is an acceptable fit between model simulation and data for at least one parameter set, we keep the model and seek for predictions with low uncertainty, i.e. core predictions, for the found acceptable parameters. Core predictions can be tested experimentally, and new data is added to the estimation process. If there are no useful predictions with low uncertainty to be found, the simulation result can instead be used to decide which experiment to perform to reduce the uncertainty of the model, and thus be able to obtain core predictions in the next modeling cycle.
The hypotheses are created from prior knowledge of the signaling network and available experimental observations, and while testing these hypotheses we are able to draw conclusions in the form of rejected models and predictions with low uncertainty, i.e. core predictions [13-15]. Rejected models are models that are not sufficient to explain all experimental observations. However, more complex models might be sufficient. We thus use the rejected models to build on when we create new hypotheses to test. On the other hand, we use conclusions in the form of core predictions as a director in the decision of which new experiments to perform to gain further information about the system. In the following sections, the basic concepts and notations of our mathematical modeling approach are explained in detail.

ORDINARY DIFFERENTIAL EQUATIONS AND OTHER MODELING FRAMEWORKS

Models based on ordinary differential equations (ODEs) describe dynamic behaviors and are ideal to simulate time resolved data. ODE models include states that correspond to amounts or concentrations of substances such as specific signal mediating proteins; reaction rates that correspond to the dynamic transition between the states; parameters that correspond to kinetic rate constants, initial conditions, or scaling factors; and output variables that correspond to the experimental observations.

As an example, the simple model of insulin receptor activation by insulin (Figure 3) can be described with the following system of ODEs:

\[
\begin{align*}
\frac{d}{dt}(IR_m) &= -v_{1a} - v_{1b} + v_{1f} + v_{1e} \\
\frac{d}{dt}(IR_{ins}) &= v_{1a} - v_{1b} \\
\frac{d}{dt}(IR_m_{YP}) &= v_{1b} + v_{1b} - v_{1c} - v_{1f} \\
\frac{d}{dt}(IR_i_{YP}) &= v_{1c} - v_{1d} \\
\frac{d}{dt}(IR_i) &= v_{1d} - v_{1e} \\
\frac{d}{dt}(X) &= v_{2b} - v_{2a} \\
\frac{d}{dt}(Xp) &= v_{2a} - v_{2b}
\end{align*}
\]

where \(IR_m, IR_{ins}, IR_m_{YP}, etc\) are the states and \(v_{1a}, v_{1b}, v_{1f}, etc\) are the reaction rates (Figure 3). These rates are described by equations that depend on the states and kinetic parameters \((k_{1a}, k_{1b}, etc)\), and the input parameter \((\text{insulin})\):

\[
\begin{align*}
v_{1a} &= IR_m \cdot k_{1a} \cdot \text{insulin} \\
v_{1b} &= IR_{ins} \cdot k_{1b} \\
v_{1c} &= IR_m \cdot k_{1c} \\
v_{1d} &= IR_{ins} \cdot k_{1d} \\
v_{1e} &= IR_m \cdot k_{1e} \\
v_{2a} &= IR_{ins} \cdot k_{2a} \\
v_{2b} &= IR_m \cdot k_{2b}
\end{align*}
\]
The output variable in this simple model is the total amount of phosphorylated receptor scaled with a parameter for normalization to data:

\[ \text{output} = k_{\text{scale}} \cdot (\text{IRm}_Y + \text{IRi}_Y) \]

To simulate the model all the kinetic and scaling parameter values must be specified, as well as the initial conditions of all states. These values are typically unknown, and have to be estimated (see next section: Parameter estimation).

Figure 3: Outline of the simple insulin receptor activation model with a negative feedback
Arrows represent reactions between states; dashed arrows represent activation of a reaction. The model states are: IRm (inactive insulin receptor in plasma membrane), IRins (insulin bound IRm), IRm_Y (tyrosine phosphorylated IRm), IRi_Y (internalized and tyrosine phosphorylated IR), IRi (internalized inactive IR), X (inactive feedback protein), X_P (active feedback protein).

There are situations when the ODE modeling framework is not the appropriate choice. For example when it comes to high-throughput data without time resolution, there are other modeling frameworks that are better suited (reviewed in [7]). The simplest modeling frameworks are entirely data-driven and consist of methods from statistics (for example clustering and principal component analysis). Also, modeling frameworks based on
Bayesian inference use statistical methods to calculate the most probable network of interactions between the measured variables [16, 17]. There is another category of modeling frameworks that is based on logic relations, like Boolean or Fuzzy boolean frameworks (reviewed in [18]). The logic model structures are also simple compared to ODE models, and do not include kinetic parameters to determine. Finally, there are model formulations that are more complex than ODE models, such as partial differential equations, which contain spatial resolution, and stochastic models, which describe random processes.

PARAMETER ESTIMATION

The values of model parameters in ODE models (for example kinetic rate constants) are hard to determine experimentally for most biological systems. Regarding parameters for the insulin signaling system, there have been efforts to measure and calculate parameters for the binding between insulin and the insulin receptor (IR) [19]. However, such detailed knowledge is not available for human adipocytes. Also, parameters for downstream insulin signaling have not been measured. We instead estimate values of the parameters using the model structure, the experimental observations, and somewhat realistic limits of the parameter values. We use an optimization algorithm to test different parameter values within the limits, and evaluate the agreement between the simulated output of the model and the observations using the sum of squares of the residuals weighted by the standard error of the mean (sem). We use a function in the Systems biology toolbox for Matlab [20] – simannealingSBAO – that combines a simulated annealing [21] with a downhill simplex algorithm [22]. Simulated annealing is designed not to find the global optimum, but a good enough approximation of that optimum in a large parameter space. In the design of the combined algorithm, worse solutions are accepted in the beginning of the optimization process, but the probability of accepting such solutions decrease with time according to a manually defined scheme. We use a modified version of the simannealingSBAO function that initiates multiple simplexes that are situated far away from each other in the space of parameters. With the multiple simplexes we are able to find multiple areas in the space of parameters – areas which potentially include additional acceptable parameters [13, 23].

We save all acceptable sets of parameters, i.e. all sets of parameters that together with the model produce a simulation that is in good agreement with the data according to a statistical \( \chi^2 \) test. If no such set of parameters is found, the model is rejected. If at least one such set of parameters is found, we usually find thousands of slightly different as well as substantially different sets of acceptable parameters. To be able to display these thousands
sets of parameters, we pick the extreme acceptable parameter sets, i.e. the parameter sets that contain a maximal or minimal value. Therefore, if a model contains 12 parameters, there are 12 maximal and 12 minimal values (24 sets of parameter values). Among the acceptable parameters, we also pick the best and worst solutions, i.e. the maximal and minimal value of the sum of squares of the residuals (2 sets of parameter values), since these can give additional information that is interesting to display.

Simulations with the found acceptable parameters can be used to draw conclusion, or to decide upon new experiments to perform to reduce uncertainty in model predictions. Such experiments are usually focused on reducing the largest uncertainties in the model by measuring states or combination of states with large uncertainty in model predictions.

CONCLUSIONS: REJECTIONS AND CORE PREDICTIONS

We use two different conclusions as directions in the model/data analysis process: rejections and core predictions (Figure 2). A rejection of a model means that the model is not good enough to describe the available data. A rejection of a model is a final statement, as opposed to a statement that a proposed model can describe data. In other words, a proposed model is acceptable only until new data have shown the contrary, but a rejected model will continue to be rejected also with new data. Model development thus continues as long as there are things of interest to understand and measure in the system. Core predictions – predictions with low uncertainty – are the other kind of conclusion we use in the process of analyzing data with mathematical models. In practice, core predictions are simulated predictions using a representation of all found parameter values that agree with the experimental observations (Figure 4). It is crucial to take the uncertainty of predictions into account since the system we study is underdetermined and unique estimations of the parameter values are not possible. A core prediction can be understood as a property that must be fulfilled if the evaluated model structure should serve as an explanation to data. We use core predictions in the design of new experiments.
Figure 4: Example of how to use core predictions
(A) A simple model structure with states X1-X3 and reaction rates v1-v3. (B) A representation of all found acceptable parameter sets are simulated together with the model structure (lines) and experimental data (dots ± sem) for the state X2. (C) A core prediction for the state X3 that was not measured shows that >40% of total X must be in the state X3 for this model structure to explain the data for X2. (D) A core prediction for the ratio of the rates v3 and v2 shows that v2 is always faster than v3 since the ratio is always less than 1. The core prediction in (C) can possibly be tested experimentally to be able to reject or strengthen the belief in the model structure. Different lines represent simulations of different acceptable parameter sets.

In Paper IV, we use a new method to search for specific core predictions [14, 24]. With this method, we perform new parameter estimations where we search for sets of parameters that extend the core prediction as far as possible, with the side condition that there is still a good enough agreement with experimental data (Figure 5). This approach is valuable since alone the optimization algorithm possibly will miss acceptable solutions for complex optimization problems. The missed solutions in the direction of the core prediction are
crucial to find before performing new experiments, since we use core predictions as conclusions. These solutions are found more easily with this method.

**Figure 5: Methods to search for a specific core prediction**

(A) A simple model structure with states X1 and X2 and reaction rates v1 and v2, where X2 is degraded and thus the total amount of X is decreasing over time. (B) Experimental data (dots ± sem) and model simulations (lines) of X2. (C) To be able to fit the simple model with the X2 data, the total amount of X at 90 minutes must be < 60% of starting total X (grey area) for all different acceptable parameter simulations (lines). The different lines represent a search for increasing values of total remaining X at 90 minutes but still with acceptable agreement to original X2 data. (D) The simulations of total remaining X at 90 minutes compared with agreement to original X2 data (dots). Blue lines intersect at 41, which is the $\chi^2$ value for rejection, and 60, which is the % remaining X at 90 minutes.
THE INSULIN SIGNALING NETWORK

The hormone insulin regulates levels of both glucose and fatty acids in the circulation [25]. Under conditions of elevated blood glucose levels, insulin causes liver cells (hepatocytes), muscle cells (myocytes), and adipocytes to take up and store glucose as glycogen. Insulin also inhibits the release of the hormone glucagon and thereby inhibits the release of glucose from the liver. Other systemic effects of insulin are stimulation of amino acid uptake, protein and lipid synthesis, and inhibition of lipolysis.

Figure 6: A simplified view of insulin signaling in adipocytes without feedbacks and crosstalk
In response to insulin several proteins are activated and intracellular processes are initiated. These processes are for instance nuclear transcription, glucose uptake, and protein translation.

The intracellular insulin signaling network (reviewed in [26, 27]) is controlled through the binding of insulin to the insulin receptor (IR). Downstream signal mediators, such as the insulin receptor substrate-1 (IRS1), are recruited to IR followed by activation though phosphorylation at tyrosine (Tyr) residues. IRS1 is also phosphorylated by protein kinases...
at serine (Ser) and threonine (Thr) residues that serve as negative or positive modifiers of insulin signaling. The subsequent signaling cascades involve phosphoinositide 3-kinase (PI3K), phospho-inositide-dependent kinase-1 (PDK1), protein kinase B (PKB), and mammalian target of rapamycin (mTOR) (Figure 6). Insulin signaling results in several intracellular responses such as nuclear transcription, protein translation, and glucose uptake.

INSULIN BINDING TO THE INSULIN RECEPTOR

The insulin receptor (IR) is a transmembrane protein with two extracellular α-subunits that bind insulin molecules and two transmembrane β-subunits with cytosolic protein kinase activity [28]. Upon insulin binding, IR autophosphorylates on Tyr residues [29], which results in IR kinase activity towards other substrates, and a downstream signaling cascade is thereby initiated (Figure 7).

Figure 7: The insulin receptor
Insulin binding to the insulin receptor α-subunits result in autophosphorylation of the β-subunits.

The IR-insulin binding has been studied in competition and dissociation experiments to unravel the characteristics of IR and the mechanisms of binding and activation [19]. The data have been analyzed using mathematical modeling approaches and the important finding are: i) several insulin molecules can bind to each receptor [19, 30-33], ii) IR contains heterogeneous binding sites with different affinities for insulin [19, 29-31, 34-36], and iii) these different affinities can be explained by a specific interaction between the two α-subunits after insulin binding [19, 30, 31, 36]. Presently, the only mathematical model that can simulate all complex characteristics in insulin binding data is a comprehensive model by Kiselyov et al. [19] that is based on advanced biochemical structural information about IR. We have linked the Kiselyov insulin binding model [19] with our own developed
models of downstream insulin signaling (Paper I). Insulin-IR binding experiments are mostly performed in cell lines, but there are also some limited data from human adipocytes. The insulin receptor of human adipocytes have a high affinity for insulin [37]. Also, human adipocytes show no detectable IR degradation during the first 45 minutes of insulin stimulation [38].

INSULIN RECEPTOR INTERNALIZATION AND ACTIVATION OF IRS1

After insulin binding and activation the insulin receptor will be rapidly internalized into the cell via small invaginations of the plasma membrane (caveolae) [39] (Figure 8). The internalized IR dissociates from insulin and dephosphorylation occurs before the inactive form of IR is recycled back to the plasma membrane [40, 41]. The internalization and recycling of IR have been studied using mathematical modeling approaches in [13, 42-48]. The insulin receptor internalization and recycling are involved in creating an overshoot in the insulin response which is observed in IR autophosphorylation as well as in the downstream phosphorylation of IRS1 [13, 42]. This overshoot in the insulin response have been thoroughly studied in human adipocytes with a combined experimental and mathematical modeling approach [13, 42]. Different feedback mechanisms, such as insulin degradation and internalization, were proposed as explanations for the observed overshoot in the insulin response, but no single feedback mechanism could explain all observations. However, IR internalization was shown to be a necessary feedback mechanism. A mathematical model involving receptor internalization in combination with another feedback, which results in dephosphorylation of internalized IR, was proposed and that model could explain all observations [13]. We used this final proposed mathematical model for IR-IRS1 in the minimal model analyses in Paper I and II and further developed the model in Paper III.

In Paper II, we studied a similar overshoot in the insulin response at the level of IR and IRS1 in data from rat adipocytes. In rat adipocytes, IRS1 is not co-localized with IR in caveolae in the absence of insulin, as is the case in human adipocytes [49, 50]. We discovered that mathematical models describing data from rat adipocytes also need internalization of IR to explain data. However, the IR-IRS1 phosphorylation dynamics in rat adipocytes was different from the human data. In rat adipocytes, the peak of IRS1 phosphorylation apparently occurs earlier that the peak of IR phosphorylation, which is a counter-intuitive behavior since IRS1 is phosphorylated downstream of IR. In turned out that that this counter-intuitive behavior can be explained simply by looking at the data in
another way. If the IRS1 response to insulin is decreasing before the IR response to insulin, it will look like the IRS1 response occurs earlier than the IR response even if the increase in response is simultaneous for IR and IRS1, because the response is normalized to the respective maximal values.

Figure 8: Insulin receptor internalization
After insulin binding and activation of the insulin receptor, small invaginations (caveolae) containing the insulin receptor will release from the plasma membrane.

In Paper II, we used a conclusive mathematical modeling approach and rejected explanations that were not sufficient to describe the reversed order of phosphorylation. With this approach we concluded that two pools or phosphorylated IR – a faster responding pool in the plasma membrane and a slower responding internalized pool – could explain this observation. The faster responding pool transmitted a fast signal to IRS1 while the slower pool was the dominating IR pool. However, this explanation required a large pool of internalized receptors, which we have previously shown is not the case [39]. A modified two pool hypothesis with limiting IRS1 concentration and specific IR-IRS1 binding could explain the reversed order of peaks of IR and IRS1. We also concluded that internalization together with a negative feedback downstream of IRS1 to the phosphorylation of IRS1 could explain the reversed order of peaks and also all our available phosphorylation and internalization data from rat adipocytes. This negative feedback model for rat adipocyte IR-IRS1 signaling in combination with the human adipocyte IR-IRS1 model in [13] provided an even better fit with rat adipocyte data (Paper II). The combined model can thus simulate IR-IRS1 signaling in both human and rat adipocytes, using only a change in parameter values.
Phosphorylation of PKB by PDK1 and mTORC2

Phosphorylated IRS1 activates PI3K that increases the concentration of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) in the plasma membrane. PIP3 continues the signaling cascade by recruiting PKB and PDK1 to the plasma membrane [51] where PDK1 phosphorylates PKB at Thr 308 [52, 53]. Also, mTOR complex 2 (mTORC2), phosphorylates PKB at Ser 473 [54]. Both phosphorylations are needed in order for PKB to be fully active and specific to its substrates [55]. Targets of PKB include glycogen synthase kinase-3 (GSK3), which is involved in control of glycogen synthesis, and AS160, which is involved in the control of glucose uptake [56] (Figure 9).

**Figure 9: Phosphorylation of PKB**

Phosphorylation of PKB by PDK1 at Thr 308 and by mTORC2 at Ser 473 are both needed for PKB to receive full kinase activity. Important targets of active PKB are the glycogen synthesis kinase GSK3, AS160 involved in glucose transporter translocation, mTORC1 which is a signaling hub involved in many cellular processes, and the transcription factor FOXO.

mTORC2 is a protein complex that consists of several proteins including mTOR and Rictor. Knockout mice lacking the Rictor protein (thus lacking mTORC2) in the adipose tissue have larger organs that normal mice and raised levels of insulin-like growth factor-1 (IGF-1) indicating a central role of mTORC2 in adipose tissue in control of systemic growth and metabolism [57]. Phosphorylation of mTOR at Ser 2481 have been shown to be
specific for mTORC2 [58]. But the dynamics of mTOR Ser 2481 phosphorylation is different from the dynamics of PKB phosphorylation at Ser 473 in the HeLa cell line [59]. The authors use a mathematical modeling approach and cannot simulate PKB Ser 473 phosphorylation data using inputs only from mTORC2, but need to include an extra unknown PKB Ser 473 kinase “PDK2”. However, the authors constrain their model to a strict ordering of phosphorylations at PKB, i.e. the Ser 473 phosphorylation is only allowed to occur after the Thr 308 phosphorylation. This strict order of phosphorylation is not supported by the literature, instead the two phosphorylations seem to be independent of each other [60]. Without this constrain there might be another solution were no longer the “PDK2” kinase is needed for the model to explain the PKB Ser 473 phosphorylation data. Another potential issue with the measurements of mTORC2 activity in [59] is that the specificity for mTORC2 phosphorylation of mTOR at Ser 2481 is questionable [61]. This phosphorylation may occur both in mTORC1 and mTORC2.

In the development of our insulin signaling model in Paper III, we implement phosphorylation of PKB at Thr 308 and Ser 473 independent of each other. The phosphorylation of PKB at Thr 308 via IRS1 and phosphorylation of PKB at Ser 473 via mTORC2 is enough to describe our data for normal subjects reasonably well.

REGULATORY FEEDBACKS CONTROL INSULIN SIGNALING VIA IRS1

The insulin signal is controlled with positive and negative regulations via several different protein kinases that phosphorylate Ser and Thr residues of IRS1. The protein complex mTORC1 is involved in this regulatory feedback signaling (reviewed in [62]). mTORC1 has a broad kinase activity and phosphorylates S6 kinase (S6K), involved in control of protein translation, as well as many other substrates with different affinities [63]. mTORC1 is specifically inhibited by the drug rapamycin. 3T3-L1 cells (a mouse derived fibroblast cell line differentiated to adipocyte-like cells) treated with rapamycin show decreased insulin stimulated IRS1 phosphorylation at several Ser phosphorylation residues including Ser 307 [64, 65]. The same decreased phosphorylation of IRS1 at Ser 307 in response to rapamycin treatment is also seen in vivo in rat muscle and adipose tissue (corresponding residue in rat and mice is Ser 302) [64]. Also in isolated human adipocytes, insulin-stimulated phosphorylation of IRS1 at Ser 307 is affected by rapamycin treatment [66, 67]. The phosphorylation of IRS1 at Ser 307 has been suggested to occur downstream of S6K, but we have shown that it is unlikely that S6K is the kinase for phosphorylation of IRS1 at Ser 307, at least in human adipocytes [68]. We have observed a positive effect of
phosphorylation of IRS1 at Ser 307 on the phosphorylation of IRS1 at Tyr in human adipocytes: rapamycin reduces also the phosphorylation of IRS1 at Tyr [66]. There are contradicting reports from experiments in cell lines, where phosphorylation of IRS1 at Ser 307 has a negative effect on insulin signaling [69]. However, experiments in cell lines have also shown that replacement of Ser 307 with alanine reduced the insulin-stimulated Tyr phosphorylation of IRS1 and reduced the insulin-stimulated phosphorylation of S6K [70], indicating a positive effect of the Ser 307-phosphorylation on IRS1 phosphorylation at Tyr residues and therefore on downstream signaling.

Ser 307 (Ser 307 in rat and mice) is another IRS1 residue phosphorylated downstream of mTORC1 [71] that has been well studied (Figure 10). Phosphorylation of this residue seemed to have a negative effect on IRS1 phosphorylation at Tyr by blocking interactions between IR and IRS1 and thus inhibiting insulin signaling [72]. However, knock-in mice with IRS1 Ser 312 replaced with alanine have impaired muscle insulin signaling [73] indicating a positive role in insulin signaling of phosphorylation of IRS1 at Ser 312 in vivo in mice.

In our comprehensive dynamic model of insulin signaling (Paper III-IV), we include phosphorylation of IRS1 at Ser 307 directly downstream of mTORC1 (Figure 10). We implement this phosphorylation to be independent of the phosphorylation of IRS1 at Tyr.

Figure 10: Feedbacks from mTORC1 to phosphorylation of IRS1 at Ser 307 and Ser 312
Phosphorylation at these residues have been shown to have positive and negative effects on the level of IRS1 phosphorylation at Tyr residues and hence the intracellular signaling.
and therefore we do not constrain the model parameters for Ser 307 phosphorylation to have a positive or negative effect on phosphorylation of IRS1 at Tyr. The best fit with data is obtained with a positive effect from IRS1 Ser 307 to Tyr phosphorylation (Paper III).

**GLUCOSE TRANSPORTER TRANSLOCATION IN RESPONSE TO INSULIN TO ENHANCE GLUCOSE UPTAKE**

The molecular regulation of glucose transport over the plasma membrane by insulin occurs in several steps: translocation of GLUT4 vesicles from intracellular storage compartments to the cell surface, tethering, i.e. the first interaction with the plasma membrane, docking of vesicles, fusion where the vesicles merge with the plasma membrane, and finally removal of GLUT4 from the cell surface (reviewed in [74]). The knowledge of these steps has evolved during the years and mathematical models of the translocation process have been used as tools to understand data for more than 20 years [75-82]. Quon [76] presented a simple two pool, two parameter model based on observations of glucose transporter translocation in rat adipocytes. Holman et al. [77] showed that at least three pools of GLUT4, including two intracellular pools, was needed to simulate all available GLUT4 translocation data from rat adipocytes and 3T3-L1 adipocytes. With four or more pools of GLUT4 the authors obtained a transient simulation of GLUT4 appearance at the cell surface [77]. Later, a combined experimental and modeling approach [80] discovered that not all intracellular GLUT4 is ready to be released to the plasma membrane and showed that there are different responses to insulin for the different pools [80]. Stenkula et al. [81] studied transfected rat adipocytes and found single GLUT4 molecules, but also functional clusters of GLUT4, in the plasma membrane. These clusters were introduced in the Holman model [77], and the combined dynamic model is in agreement with the presented dynamic data of GLUT4 vesicle fusion events and simulates the transient behavior of GLUT4 appearance [81].

AS160, a substrate of PKB, is involved in transmission of the insulin signal to translocation of GLUT4-containing vesicles in the cytosol to the plasma membrane [83, 84] (Figure 11). To unravel which steps of the translocation process that are regulated by AS160, Brewer et al. [82] silenced AS160 in 3T3-L1 adipocytes combined with inhibitors of PKB and found that a PKB substrate other that AS160 regulates the transfer from a non-cycling GLUT4 pool to a cycling pool and that AS160 regulates the reverse action. These results were tested and confirmed with mathematical modeling.
The translocation of GLUT4 differs between human and rat adipocytes. In human adipocytes, about 25% of GLUT4 is located in the plasma membrane in the basal state and this amount increases approximately 2-fold with insulin stimulation [85]. In rat adipocytes, there is also around 50% of GLUT4 at the plasma membrane after insulin stimulation, but the basal amount is less than 5% of total GLUT4 [86]. Also in 3T3-L1 adipocytes the GLUT4 content at the plasma membrane increases more than 10-fold with insulin due to low basal content [87].

Figure 11: Mechanisms of glucose transporter translocation
Translocation of the insulin-regulated glucose transporter (GLUT4) from cytosolic vesicles to the plasma membrane upon insulin stimulation is mediated via PKB and AS160.

Our developed models of glucose uptake in Paper I and Paper III-IV contain no detailed mechanisms of glucose transporter translocation, since we do not have detailed information about these mechanisms from human adipocytes. Instead AS160 simply causes GLUT4 to translocate to the plasma membrane where glucose uptake is proportional to the amount of GLUT4 molecules present and to the glucose concentration used in the experiment, with saturation applied to the glucose concentration. We use the steady state observations of GLUT4 content in the plasma membrane [85] and our own measurements of glucose uptake for different concentrations of insulin and glucose (Paper I) to find values of the parameters. We also add a constant GLUT1 glucose uptake, since the insulin-insensitive GLUT1 glucose transporter also is present in adipocytes. GLUT1 is responsible for the basal glucose uptake required for the basal needs of the adipocytes and other cells. The contributions of GLUT1 and GLUT4 to glucose uptake can be examined with a drug that mostly inhibits the GLUT4 contribution (Indinavir), which has been shown in cell lines
We tried to access this drug to perform such measurements for human adipocytes for the analysis in Paper I, but without success.

INSULIN CONTROL OF ERK SIGNALING

Extracellular signal-regulated kinases 1/2 (ERK), members of the Map kinase family of protein kinases, are multifunctional kinases with activities both in the cytosol and the nucleus of cells. Upon growth factor stimulation, receptor Tyr kinases activate Ras/Raf, and the dual specificity kinases MEK1/2 that phosphorylates ERK at Tyr and Thr residues [89] (Figure 12). The phosphorylation of ERK by MEK1/2 occurs in the cytosol of cells [90]. Phosphorylated ERK can translocate to the nucleus to phosphorylate its nuclear targets [91]. One such target is Ets-like gene 1 (Elk1) [92], a nuclear localized transcription factor that is phosphorylated at multiple residues, whereof Ser 383 and Thr 363 have been shown to be important for transcriptional activity [93, 94]. The ERK signaling branch is highly activated by growth factors like the epidermal growth factor (EGF), but is also activated to a smaller extent by insulin [95, 96]. The insulin stimulated phosphorylation of ERK occurs via the proteins Shc [97] and/or IRS1, which both are phosphorylated by the insulin receptor in response to insulin [98] (Figure 12).

To be able to further develop the insulin-signaling model and include the ERK pathway, we measured the dynamic response to insulin for ERK phosphorylation (Paper IV). We found a maximal response after 10 minutes and that the signal is back to basal levels at 60 minutes in human adipocytes. In differentiated preadipocytes from human subjects, the first 15 minutes after insulin stimulation have been studied [99]. The authors found a maximal insulin stimulation of ERK phosphorylation at 10 minutes. The same ERK dynamics have also been studied in 3T3-L1 adipocytes that have a higher response to insulin (up to 18-fold) [100]. ERK phosphorylation in this cell line is maximal at 2 minutes. It is also interesting to note that there are differences in the ERK dynamics between different tissues stimulated in vivo with insulin in mice [101]. While ERK phosphorylation in muscle tissue continues to increase during 30 minutes, the adipose tissue ERK phosphorylation return to basal levels [101]. In Paper IV, we tested different hypotheses to explain our findings of the insulin stimulated ERK dynamics in human adipocytes.

In 3T3-L1 adipocytes it has been shown that ERK is activated by insulin downstream of PI3K, since the insulin response is reduced with addition of wortmannin, a specific PI3K inhibitor [102]. The same finding is also reported from rat adipocytes stimulated with insulin and inhibited with wortmannin [103-105]. One of the reports, [103], also show that
the Shc pathway is not involved in the activation of ERK in response to insulin in rat adipocytes. Instead the IRS1/PI3K pathway is exclusively used for ERK activation in these adipocytes. This finding is in contrast to findings in some cell lines where the Shc pathway have been shown to be involved in the activation of ERK in response to insulin [106, 107]. We have previously shown in human adipocytes that activation of ERK and Elk1 is downstream of IRS1 since the expression of a mutant IRS inhibits Elk1 response to insulin [108]. However, to be able to fit with all data of phosphorylation of ERK, we included the ERK signaling branch downstream of both IR and IRS1 in our developed insulin signaling model in Paper IV.

Figure 12: Insulin stimulated ERK phosphorylation
The phosphorylation of ERK after insulin stimulation is mediated via the proteins Shc and/or IRS1 that both are associated to the insulin receptor. The subsequent signaling cascade involves Ras, Raf, MEK, and ERK.

Stimulation with different growth factors gives rise to different dynamic behaviors of ERK (reviewed in [109, 110]), and these observations have been analyzed with mathematical models. Brightman and Fell [111] showed that inhibition of a negative feedback in the pathway can explain that ERK phosphorylation in the same cell type can be transient or sustained depending on the growth factor used for stimulation. Also, different cell types stimulated with the same growth factor give rise to different dynamic behaviors [112]. Kiel and Serrano [112] have shown with mathematical models that different feedback-strengths
in different cells could explain these observations. Feedback loops in the ERK signaling network have also been studied to bring light upon other biological phenomena using mathematical modeling approaches [113, 114]. The studied biological phenomena ranges from robustness [115, 116] and oscillations [117-120], to cooperativity [121] and ultrasensitivity [117, 122]. Ultrasensitivity, i.e. that the response to increasing concentrations of growth factor becomes more switch like further down in the signaling cascade [123], was theoretically predicted in ERK signaling by Huang and Ferrell [124] using a mathematical modeling approach. The authors also confirmed this prediction experimentally using extracts of egg cells (xenopus oocytes). Levchenko et al. [125] built on the Huang and Ferrell model to study the effect of addition of scaffolding proteins to the model. Scaffolding proteins bind to several signaling intermediaries and are important in ERK signaling since they can facilitate signal transduction, segregate signaling pathways from crosstalk, and target signals to specific locations in the cell [126, 127]. In the hypothesis testing in Paper IV, we found that scaffolding, but not a simple feedback mechanism, could explain the dynamic ERK phosphorylation pattern in human adipocytes (Figure 13).

In Paper IV, we also tested other hypotheses that were compatible with the dynamics of the ERK phosphorylation data (Figure 13). One of the hypotheses dealt with the dual phosphorylation of ERK by MEK. This dual phosphorylation process has previously been modeled with so called processive and distributive mechanisms [128-130]. In a distributive phosphorylation mechanism MEK binds to ERK and phosphorylates one of the residues, and then MEK releases single phosphorylated ERK. MEK also binds to single-phosphorylated ERK and phosphorylates the second residue, and then releases dual-phosphorylated ERK. In a processive phosphorylation mechanism, on the other hand, MEK binds and phosphorylates ERK at one residue and then phosphorylates the other residue before dissociation from ERK. Mathematical analysis has revealed that the phosphorylation of ERK is distributive in experimental systems where proteins have been extracted from cells [128, 131]. It has also been shown with mathematical models that addition of crowding agents to the media, to mimic intracellular conditions, make the phosphorylation of ERK processive [132, 133]. However, in cell line experiments combined with mathematical modeling, phosphorylation of ERK was shown to be distributive in response to the cytokine erythropoietin [134]. We tested dual phosphorylation with different mechanisms in Paper IV, and found a simple processive model that could account for the dynamic data from human adipocytes.

Finally, nuclear translocation of ERK could also explain the dynamics of ERK phosphorylation (Figure 13). Fujioka et al. [135] have studied the details of nuclear translocation of ERK in HeLa cells with experiments and modeling. Using fluorescent
probes, the authors measure interesting parameters of ERK activity, i.e. rate of phosphorylation and dephosphorylation, rate of nuclear import and export, and rate of binding and dissociation with MEK. With these parameters, the authors create an interesting simulation model of ERK signaling and compare the results with independent data. The authors also compare their measured parameter values with values reported in other modeling papers and show major differences in some of the parameters [135]. These differences are probably due to differences in cell type and/or experimental setup and highlight the importance of using data from the same cell type in the development of mathematical models.

Figure 13: Hypothesis testing and a common minimal model

Three hypotheses explain the dynamic ERK phosphorylation data: scaffolding, dual phosphorylation, and nuclear translocation. These hypotheses can all be simplified to the same minimal model.

All the acceptable hypotheses for the dynamics of ERK phosphorylation (Paper IV) shared the same features and could be reduced to a common minimal model (Figure 13). The common minimal model includes sequestration of non-phosphorylated ERK and the analysis of the model revealed that more than 40% of total ERK had to be in this sequestered pool. We studied total ERK protein with immunofluorescence confocal
microscopy to study the location of this sequestration in the adipocyte and found that ERK concentration was high in the nuclear regions. We could, however, not see an insulin effect on this ERK sequestration.

There are also a few famous detailed models of ERK signaling in the literature (reviewed in [136]). Kholodenko et al. [137] have developed a mathematical model of the transient EGF response of the upstream signaling intermediaries, and continued to develop the model with more data, also for different temperatures during stimulation [138]. Schoeberl et al. [139] also developed an EGF response model. This detailed model with 94 states and 95 parameters, which mostly have been taken from literature, was compared with data for transient phosphorylation of ERK. These two detailed models for ERK signaling were not used to draw conclusions, but to include all known interactions and gather the available knowledge of connections between intermediaries in the ERK signaling network.

Despite all available models of ERK signaling, there are only a few mathematical models for insulin control of the ERK signaling pathway [96, 140, 141]. Borisov et al. [96] have studied interactions between insulin- and EGF-stimulated ERK signaling. Aldridge et al. [140] have studied insulin and EGF, but also tumor necrosis factor (TNF), involved in inflammation, and focus on the crosstalk between these signaling pathways. Recently, a pure theoretical attempt at modeling the complete insulin signaling network, including ERK, was published [141]. None of these models were based on dynamic data from human adipocytes, and we could not use them in the development of the extended insulin signaling model in Paper IV.

**Detailed Mathematical Models of the Insulin Signaling Network**

An important first mathematical model of the insulin signaling network, from insulin-IR interactions to GLUT4 translocation, was published more than a decade ago [142]. In this first detailed model of the metabolic part of insulin signaling, Sedaghat et al. combined previous models of insulin-IR binding and IR recycling [33, 44] with models of GLUT4 regulation [75, 76] and knowledge about mechanisms and intermediates involved in downstream signaling to control of glucose uptake, i.e. phosphorylation of PI3K and PKB, and GLUT4 translocation [142]. Sedaghat et al. used model parameters from the literature and compared the ODE model simulations with steady-state dose-response data from rat adipocytes for some of the signaling intermediaries. However, the lack of time-resolved data is problematic since the authors claim that they describe the dynamics of the insulin
signaling network. Despite the shortcomings of the Sedaghat model, it has been extensively used and also further developed [143-145] and analyzed [146]. Recently, another more theoretical attempt at constructing a detailed model of insulin signaling was published [141]. The authors have formulated simple equations for a sigmoidal activation to simulate dose-response behaviors and compare with data from different sources. Again, in [141] the lack of dynamic data is a limitation of the study.

Figure 14: The detailed insulin signaling model
The insulin signaling model developed in Paper III-IV contains 32 states (IRm, IRm-YP, etc), 41 reactions (v1a-v11b, bold arrows), and 49 parameters. Regular arrows show where activation occurs in the model. Red numbers show the three diabetes parameters: reduced IR concentration, reduced GLUT4 concentration, and reduced mTORC1-derived feedback to IRS1. From Paper IV.

In contrast, a modeling approach of the insulin signaling network in HeLa and C2C12 cell lines has used dynamic data for many signaling intermediaries [59]. The authors focus on the activation of mTORC2 and conclude by hypothesis testing that the activation is not
downstream of PKB, or IRS1, but probably downstream a PI3K that is not effected by the mTORC1-derived feedback to IRS1. The conclusions in [59] depend on the authors confidence in a negative feedback from mTORC1 via S6K to IRS1 phosphorylation, which, at least in human adipocytes, is not important (Paper III). Instead we show that a positive feedback from mTORC1 to phosphorylation of IRS1 at Tyr via Ser 307-phosphorylation of IRS1 is reduced in T2D (Paper III).

With Paper III, we are now able to present a comprehensive ODE model of the insulin signaling network based on extensive experimental data from human adipocytes gathered in our laboratory for several years. The experimental data are both time resolved and dose dependent and gathered for many important signaling intermediaries such as IR, IRS1, PKB, AS160, glucose uptake, and also S6K and S6 as a direct measure of mTORC1 activity. With the presented mathematical model in Paper III we have taken the knowledge of dynamic intracellular insulin signaling to a new level. In Paper IV, we continue to develop this mathematical model and include the ERK signaling pathway and an important signaling contribution from ERK to S6 (Figure 14).

**EXPERIMENTAL PERTURBATIONS IN MODEL DEVELOPMENT AND VALIDATION**

Perturbations of the signaling are important to enhance and complete understanding of the insulin signaling network. Commonly used perturbations at the cellular level are the use of different inhibitors that are specific to one or more protein targets. Rapamycin is a specific inhibitor of mTORC1 that we used as a validation in Paper III, and also to understand the activation of ERK signaling in Paper IV. The effects of rapamycin in human adipocytes are seen in most proteins of the insulin signaling network (Table 1), because of the feedback from mTORC1 to phosphorylation of IRS1 at Ser 307. Rapamycin has been reported to lower glucose uptake in human adipocytes [67], although also the contrary has been reported [147]. The previously reported feedback from IRS1 to dephosphorylation of internalized IR (first described in [13]), which is included in the models in Paper III and Paper IV, seems to be important since the phosphorylation of IR is experimentally effected by rapamycin [67]. This reduction is indeed predicted by the models (Paper III).

In renal samples from humans treated orally with rapamycin, the phosphorylation of PKB at Ser 473 was enhanced and the phosphorylation of IRS1 at Tyr was lowered in response to insulin [148]. The oral rapamycin treatment also gave rise to systemic insulin resistance, as measured by enhanced 2 hour plasma glucose and insulin levels [149].
Table 1: Rapamycin effects on insulin stimulated protein phosphorylations

The arrows represent a negative effect (↓), no significant effect (→), and a positive effect (↑) of rapamycin on the measured protein phosphorylations in human adipocytes.

<table>
<thead>
<tr>
<th>Measured protein phosphorylation</th>
<th>Effect of rapamycin</th>
<th>References</th>
<th>Effect of rapamycin in the mathematical models in Paper III-IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR (Tyr)</td>
<td>↓</td>
<td>[67]</td>
<td>↓</td>
</tr>
<tr>
<td>IRS1 (Tyr)</td>
<td>↓→</td>
<td>[66, 67]</td>
<td>↓</td>
</tr>
<tr>
<td>IRS1 (Ser 307)</td>
<td>↓</td>
<td>[66, 67]</td>
<td>↓</td>
</tr>
<tr>
<td>PKB (Thr 308)</td>
<td>→↑</td>
<td>[67, 147]</td>
<td>↓</td>
</tr>
<tr>
<td>PKB (Ser 473)</td>
<td>→↓↑</td>
<td>Paper III, [67, 147]</td>
<td>↓</td>
</tr>
<tr>
<td>AS160 (Thr 642)</td>
<td>↓</td>
<td>[67]</td>
<td>↓</td>
</tr>
<tr>
<td>S6K (Thr 421/Ser 424)</td>
<td>↓</td>
<td>[67]</td>
<td>S6K (Thr 389)</td>
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<tr>
<td>S6 (Ser 235/236)</td>
<td>↓</td>
<td>Paper III</td>
<td>↓</td>
</tr>
<tr>
<td>ERK (Tyr 202/Thr 204)</td>
<td>→</td>
<td>Paper IV</td>
<td>↓</td>
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All the in vitro effects of rapamycin (Table 1) are compatible with our presented detailed mathematical model (Paper III), with the exception for phosphorylation of PKB in differentiated human preadipocytes where rapamycin was reported to increase the phosphorylation of PKB at Ser 473 and Thr 308 [147]. In our measurements of phosphorylation of PKB at Ser 473 (Paper III), we found no inhibition with rapamycin. The detailed mathematical model simulates a minimal decrease in phosphorylation of PKB at Ser 743 with rapamycin, compatible with our measurements (Paper III).

In Paper IV, we measured the effect of rapamycin on insulin-stimulated phosphorylation of ERK, and found no significant effect. These data were not directly compatible with our presented mathematical model where rapamycin slightly decreases the phosphorylation of ERK. However, we could not reject the model since the rapamycin data allow for a decrease in ERK phosphorylation with rapamycin. The slight reduction of ERK with rapamycin in the model is within the uncertainty of the data.

To gain further knowledge about the insulin signaling network, we have also used perturbations with substances other than rapamycin. In Paper IV, we used two different inhibitors of MEK activation (PD184352 and U0126) and found an important contribution of the ERK signaling pathway in the control of the ribosomal protein S6. Both these MEK inhibitors reduced the phosphorylation of S6, but not the phosphorylation of S6K in human adipocytes (Paper IV). We thus concluded that ERK control of S6 likely occurs via ERK-catalyzed phosphorylation of p90-RSK, which is a protein kinase for S6 (reviewed in...
This crosstalk has been suggested in rat adipocytes [151]. We selected the MEK inhibitor PD184352 as it was shown to be a specific MEK inhibitor [152], and supported the conclusion with a different inhibitor of MEK (U0126). We also combined rapamycin with PD184352 and studied the effect on the phosphorylation of S6 (Paper IV). We concluded that the effects of S6K and ERK on the phosphorylation of S6 are additive in human adipocytes.
INSULIN RESISTANCE IN TYPE 2 DIABETES

Type 2 diabetes (T2D) is characterized by impaired beta cell function and insulin resistance in adipose, muscle, and liver tissues. The systemic insulin resistance originates from impaired intracellular insulin signaling in these tissues. The defects of the intracellular insulin signaling have been shown to appear in several branches of insulin signaling and several layers of regulations are altered (reviewed in [98, 153]). Both the activity and levels of insulin signaling proteins are altered in T2D, but there are discrepancies between different studies [153]. These discrepancies could be due to methodology, or to examination of different kinds of cells, or to examination of material from different sub-populations.

Insulin resistance and T2D are tightly linked to obesity, and there is an increasing agreement that an expanding adipose tissue is the origin to the development of T2D [4]. In an experiment supporting this agreement, co-cultures containing differentiated pre-adipocytes obtained from insulin resistant humans and muscle cells obtained from insulin sensitive humans have shown that the insulin resistance can spread from adipocytes to other insulin responding cells [154]. Further, mice modified to have reduced GLUT4 in the adipose tissue, spontaneously develop insulin resistance also in liver and muscle tissues [155]. In a similar experiment where mice were modified to have reduced GLUT4 in the muscle tissue, glucose uptake in the adipose tissue – but not in the liver – was reduced [156].

Adipocytes are resistant to insulin prior to diagnosis of T2D, but the underlying causes to this insulin resistance remain underexplored. Suggested causes are both external factors and intracellular mechanisms. External inflammation, i.e. where macrophages infiltrate the adipose tissue and release cytokines, has been shown to correlate with insulin resistance in the adipose tissue of obese humans [157]. Another possible cause of the insulin resistance is an intracellular response to a nutrient overload. Adipocytes are quite flexible and can increase in size to store more fatty acids as triacylglycerol in the lipid droplet (Figure 1). However, they may burst if they increase in size too much since adipocyte size is positively correlated with cell death [158]. Adipocyte size is also positively correlated with insulin resistance and obesity: large adipocytes respond less to insulin than small adipocytes from the same individual [159, 160], and the mean size of adipocytes correlates with insulin
resistance as well as obesity [157, 161]. All in all these data suggest that large and insulin resistant adipocytes in obese individuals may burst due to a nutrient overload. Burst and damaged cells in the tissue attract macrophage infiltration, and indeed macrophages in the adipose tissue of obese humans are clustering around dead adipocytes [158]. The inflammation in the insulin resistant adipose tissue may therefore be a consequence – and not the primary cause – of insulin resistance, although inflammation can aggravate the insulin resistance.

mTORC1, the main regulator of cell growth, is a main hub in the insulin signaling network and has, though feedbacks, the potential to reduce the response to insulin throughout the insulin signaling network [162, 163]. Activation of mTORC1 in response to insulin has been shown to be attenuated in adipocytes from T2D patients [164]. In Paper III, we suggest a mechanism where mTORC1 senses the increased size of the adipocytes in T2D and act to attenuate the responsiveness to insulin in order to reduce further cell growth and accumulation of triacylglycerol. Along the same line, we find a reduced activation of ERK in T2D (Paper IV). This reduced activity of ERK may signal to increase adipogenesis to counteract the insulin resistance, since ERK activity can inhibit cell differentiation via peroxisome proliferator activated receptor γ (PPARγ) and fibroblast growth factor 21 [165, 166].

MECHANISMS OF INSULIN RESISTANCE IN ADIPOCYTES

The knowledge of mechanisms of insulin resistance in adipocytes has consisted of fragmented and not always compatible data, since different experimental systems have been used to gain the knowledge. The lack of compatible and dynamic data has probably been the main reason for the lack of mathematical models describing insulin resistance in adipocytes. Insulin signaling normally in adipocytes has, however, been modeled to some extent (reviewed in [12]). With this thesis we present, for the first time, a systems understanding for insulin resistance in adipocytes, based on internally consistent data.

To obtain a systems understanding of insulin resistance in adipocytes, we gathered data for critical signaling intermediaries of the insulin signaling pathway (Paper III). We found several differences between insulin signaling in T2D and insulin signaling normally. We found a reduced degree of maximal phosphorylation at steady-state for most signaling intermediaries (Figure 15). We also found a reduced sensitivity to insulin, i.e. the concentration of insulin that produces half-maximal response (EC50), such that lower insulin sensitivity results in a right-shift in the dose-response curve to higher concentrations.
of insulin and higher EC50 values. This reduced sensitivity does not appear at the level of the insulin receptor, but at the level of IRS1 and most downstream signaling intermediaries (Figure 15). In Paper IV, we complemented this knowledge with measurements of ERK signaling in T2D. Also for ERK we found a reduced absolute response to insulin and a reduced sensitivity in adipocytes from T2D patients.

Figure 15: Experimental data for insulin signaling proteins in adipocytes normally and in T2D. Blue (non-diabetic) and red (diabetic) dots and lines show measured protein phosphorylations and glucose uptake in response to different insulin concentrations. A reduced sensitivity to insulin downstream of IR was obtained for most signaling proteins and the glucose uptake. Also the dynamic response to insulin was measured, with lower response at most signaling intermediaries. P denotes phosphorylation, Y phosphorylation at Tyr residues, S phosphorylation at Ser residues, and T denotes phosphorylation at Thr residues. From Paper III.
The detailed model of insulin signaling that we developed in Paper III-IV simulates insulin signaling data from adipocytes both from T2D patients and normal controls (Figure 16). The only differences between the normal and T2D versions of the model are three clearly defined diabetes-parameters: reduced IR concentration, reduced GLUT4 concentration, and a reduced mTORC1-IRS1 feedback (Figure 14). While the reduced concentrations of IR and GLUT4 have been directly measured [167, 168], the changed feedback was a conclusion from our minimal modeling approach (Paper III). In this approach, we tested different suggested mechanisms to describe both the reduced degree of phosphorylation at all signaling intermediaries, and the reduced sensitivity to insulin downstream of IR. We concluded that suggested insulin resistance mechanisms such as reduced IR and reduced IRS1 concentrations could explain the change in the extent of phosphorylation, but not the reduced sensitivity to insulin. These mechanisms were thus rejected as single explanations. In contrast, a reduced positive feedback or an increased negative feedback to IRS1 could provide a reduced sensitivity to insulin. In the continuous model development we used the known feedback downstream of mTORC1, namely phosphorylation of IRS1 at Ser 307, as a mechanism of alteration in T2D.

Ser/Thr phosphorylations of IRS1 are commonly referred to as mechanisms causing insulin resistance (reviewed in [169-171]). These Ser/Thr phosphorylations have either negative or positive effects on insulin signaling, but negative effects are generally referred to as mechanisms of insulin resistance. For example, animal studies have showed increased phosphorylation of IRS1 at Ser 307 in liver tissue from genetically obese and overfed mice [172]. Such mice models are commonly used to study insulin resistance and T2D. Also, mice with alanine replacement of IRS1 at both Ser 307, Ser 312, and another Ser residue in muscle tissues, were more insulin sensitive that wild-type mice when fed with a high-fat diet [173]. In human adipocytes, on the other hand, phosphorylation of IRS1 at Ser 307 is reduced in T2D (Figure 15). This reduced phosphorylation of IRS1 at Ser 307 in T2D adipocytes was reversed in the presence of a Ser protein phosphatase inhibitor (okadaic acid), which also resulted in restored phosphorylation of IRS1 at Tyr [66]. The differences in IRS1 Ser phosphorylations between T2D in human verses obese mice models show the importance of using human primary cells when studying insulin resistance in T2D.

The phosphorylation of PKB in response to insulin was somewhat confusing in adipocytes from T2D patients (Paper III). Neither phosphorylation of PKB at Thr 308 nor at Ser 473 showed a substantially reduced sensitivity to insulin in T2D (Figure 15). The reduced sensitivity at the level of IRS1 was thus not transmitted to PKB, but to signaling downstream of PKB (i.e. AS160 and glucose uptake). In the model simulations we allowed for a slight reduction of the sensitivity at PKB in T2D, still compatible with data (Paper III). The magnitude of the dynamic response for phosphorylation of PKB at Thr 308 was
lowered in T2D, but was slightly higher at Ser 473. The model could not capture the elevated phosphorylation of PKB at Ser 473 in T2D (Figure 16). Interestingly, reports of an inhibitory feedback from mTORC1 signaling to mTORC2 [174, 175] is a potential explanation to the increased phosphorylation of PKB at Ser 473 in T2D. We have, however, not implemented such a feedback in our model, since these results have not been examined in human adipocytes. There have also been observations of a lower insulin response for phosphorylation of PKB at Ser 473 in adipocytes from T2D patients [176, 177].

Figure 16: Dynamic model simulations of insulin signaling normally and in T2D
The measured protein phosphorylations display different dynamic behaviors at different signaling branches. Signaling through IR-IRS1-PKB displays a fast transient overshoot behavior with an above basal steady state level. The transient behavior is slower and returns to basal levels in ERK-Elk1 signaling. Signaling downstream mTORC1 (S6K-S6) respond even slower without tendency to decrease after 60 minutes of stimulation. The diabetic state (red) has the same dynamic behavior as the normal state (blue) for all signaling proteins, but the magnitude of the response is lower in T2D.
We have previously reported that insulin has similar effects on phosphorylation of ERK in adipocytes from normal and T2D patients [178], and that there is a reduced sensitivity to insulin in the phosphorylation of ERK in T2D [179]. In Paper IV, we examined dynamics of ERK phosphorylation for the normal and T2D states and report a reduced maximal degree of phosphorylation in the T2D state, but without a difference in the fold effect of insulin between normal and diabetic state, similarly to [178]. There are also contradictory findings of an increased basal level of ERK phosphorylation, which is not significantly affected by insulin, in adipocytes from T2D patients [180]. In muscle tissue, ERK signaling is neither decreased nor increased in the diabetic state [181, 182].

LEVELS OF SIGNALING PROTEINS IN TYPE 2 DIABETES

A possible defect in insulin signaling in T2D, which could give rise to the insulin resistance, is reduced levels of some of the proteins in the insulin signaling network. For the early insulin signaling proteins, i.e. IR and IRS1, there are ambiguous results for the protein levels in the normal and T2D states. We have observed a reduction to 55 % of normal protein levels for IR in adipocytes from T2D patients [167]. However, another study reports no differences between normal and T2D protein levels of IR in adipocytes [183]. In insulin binding competition experiments, adipocytes from T2D patients bound less insulin than normal adipocytes for all tested insulin concentrations, and the authors concluded that the deceased binding was caused by a reduced level of IR and not by a change in affinity in T2D adipocytes [184, 185]. Also in muscle tissue from T2D patients there seems to be a reduction of IR protein levels as compared to muscle tissue from normal subjects [182], but the authors provide no quantification of the results.

For IRS1, on the other hand, we have reported that there is no change in protein levels in adipocytes from T2D patients compared with non-diabetic subjects [66, 167], but others report a reduction in IRS1 protein levels [180, 183]. In muscle tissue from T2D patients there was no clear reduction of IRS1 protein levels [182]. In another study, human adipocytes were treated with high insulin and high glucose to make them insulin resistant [186]. After 2 hours of treatment, insulin-stimulated glucose uptake was markedly reduced, but without reduction in IRS1 or GLUT4 protein levels. After 6 or more hours, the IRS1 protein levels were reduced, but the insulin resistance was not caused by the IRS1 reduction since the insulin resistance preceded the IRS1 reduction [186]. The contradictory findings for levels of IR and IRS1 in diabetes can be a result of different handling of cells or individual/sub-population differences. We use our results from human adipocytes [66, 167]
in the creation of the insulin signaling model in Paper III, as they have been obtained in an internally consistent manner.

For the proteins downstream of IRS1 in the insulin signaling network, there are no reported differences in protein levels in cells from normal versus T2D patients in adipose or muscle tissue, except for GLUT4 (Table 2). GLUT4 protein levels have been reported to be reduced by 50-80 % in adipocytes [168, 180, 187, 188] and by 30-50 % in muscle tissue [189] from T2D patients. In Paper III and Paper IV, we included reduced IR levels to 55 % of normal as well as reduced GLUT4 levels to 50 % of normal in the T2D state of the models.

Table 2: Reported levels of protein in adipocytes and muscle tissue in type 2 diabetes
The arrows represent less reported level of protein in T2D (↓), or no change between normal and T2D in reported level of protein (→).

<table>
<thead>
<tr>
<th>Measured protein</th>
<th>Cells/tissue samples</th>
<th>Change of protein level in T2D</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR</td>
<td>adipocytes, muscle</td>
<td>↓→</td>
<td>[167, 182, 183]</td>
</tr>
<tr>
<td>IRS1</td>
<td>adipocytes, muscle</td>
<td>→↓</td>
<td>[167, 180, 182, 183]</td>
</tr>
<tr>
<td>PI3K</td>
<td>muscle</td>
<td>→</td>
<td>[190]</td>
</tr>
<tr>
<td>PKB</td>
<td>adipocytes, muscle</td>
<td>→</td>
<td>[176, 177, 190]</td>
</tr>
<tr>
<td>mTOR</td>
<td>adipocytes</td>
<td>→</td>
<td>[164]</td>
</tr>
<tr>
<td>AS160</td>
<td>muscle</td>
<td>→</td>
<td>[189, 191]</td>
</tr>
<tr>
<td>GLUT4</td>
<td>adipocytes, muscle</td>
<td>↓</td>
<td>[168, 180, 187-189]</td>
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<tr>
<td>GLUT1</td>
<td>muscle</td>
<td>→</td>
<td>[189]</td>
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<tr>
<td>S6K</td>
<td>adipocytes</td>
<td>→</td>
<td>[164]</td>
</tr>
<tr>
<td>ERK</td>
<td>adipocytes, muscle</td>
<td>→</td>
<td>Paper IV, [180, 182]</td>
</tr>
<tr>
<td>Elk1</td>
<td>adipocytes</td>
<td>→</td>
<td>Paper IV</td>
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LINKING INTRACELLULAR INSULIN SIGNALING WITH WHOLE-BODY GLUCOSE DYNAMICS

Diseases like T2D are manifested and diagnosed at the whole-body level, but usually arise and are best treated at the intracellular level. A central function of intracellular insulin signaling in muscle and adipose tissue is to increase glucose uptake, which results in lowered levels of blood glucose. To be able to fully understand disease mechanisms of T2D, it is therefore important to relate the results from intracellular studies to the clinically interesting whole-body glucose control. Such relations benefit from mathematical descriptions, and mathematical models exist both at the intracellular insulin signaling level and at the whole-body glucose control level. However, these relations must be quantified in a sound manner; therefore we have developed an approach to link these mathematical models to create multi-level models.

THE DYNAMICS OF GLUCOSE HOMEOSTASIS

Insulin controls plasma levels of glucose by reducing production of glucose in the liver, and by increasing adipose and muscle tissue uptake of glucose (Figure 17). The dynamic interplay between glucose and insulin has been extensively analyzed with mathematical models (reviewed in [192]). The Bergman minimal model of glucose-insulin dynamics [193] was developed more than 30 years ago and is still used for applications and in research projects. This minimal model is a simple black-box model with few parameters that can be easily estimated for different populations or individuals. This minimal model is, however, not useful for linking organ or cell models, since the model lacks interpretation of the parameters in terms like “glucose uptake by insulin responding tissues”, “insulin release by pancreatic beta-cells”, etc. In recent years, a novel triple-tracer method [194] for measuring flows of glucose between organs in vivo have provided new data for creation of more detailed glucose homeostasis models. The first such model, presented by Dalla Man et al. [195], describes the systemic glucose homeostasis in an organ-based manner. The model is based on meal response measurements of glucose and insulin from more than 200 healthy human subjects. An extension of the Dalla Man model has been approved by the
American Food and Drug Administration to replace the use of animals in preclinical trials to certify new insulin pumps [196].

Figure 17: Glucose homeostasis
Glucose is absorbed from the intestine to the plasma after a meal intake. Insulin regulates the glucose release from the liver and the glucose uptake from adipose and muscle tissues to maintain normal glucose plasma levels at all times. From [6].

Creating a mathematical link using dynamic constraints

The relation between in vitro experiments with isolated adipocytes on one hand, and the adipocytes in the adipose tissue in vivo on the other hand, must be sorted out to be able to link models on these different levels. In Paper I, we developed methods with dynamic constraints from the whole-body level to create a link between our intracellular insulin
signaling models with the Dalla Man model for glucose homeostasis [195]. The dynamic constraints are limitations to the model under development (Figure 18). Input constraints are used as input to the intracellular model under development and output constraints are fitted with the output of the model and are thus used as experimental data in the optimization process (Figure 19). This use of dynamic constraints in the development of a model secures an agreement between the intracellular model and the input and output of the whole-body level model. In Paper I, we combined insulin signaling data from the intracellular level with dynamic constraints describing input and output of glucose and insulin to the adipose tissue during a meal (Figure 18). We searched for parameters for the intracellular model that both fitted our experimental \textit{in vitro} data and the dynamic constraints.

\textbf{Figure 18: Dynamic constraints are input/output limitations}

Interstitial insulin and glucose are input constraints and the glucose uptake is the output constraint to the adipose tissue model under development. Modified from Paper I.

To be able to use the adipose tissue glucose uptake profile as an output constraint, we needed to subdivide the dynamic profile of the glucose uptake in the Dalla Man model [195], because this model describes the total glucose uptake profile. The total glucose uptake profile is primarily the sum of muscle and adipose tissue glucose uptake, but it also contains the glucose uptake of the liver, the kidneys, the brain, and the red blood cells. The glucose uptake of the brain and the red blood cells is not affected by insulin and is thus easily separated from the dynamic profile. The remaining glucose uptake profile was
subdivided with the proportions 20% to adipose tissue and 80% to muscle tissue glucose uptake [197]. We ignored the liver glucose uptake since most meal glucose is directly absorbed by the liver before entering the plasma and is thus not accounted for by the Dalla Man model. Meal glucose uptake profiles in the adipose tissue have been measured and calculated with the use of tracers and blood flow measurements [198-201]. Compared to forearm (muscle tissue) glucose uptake, the adipose tissue glucose uptake seems to respond faster after the meal [199]. We used the interstitial insulin profile as provided in the Dalla Man model as one of the input constraints (Figure 18). Interstitial insulin is hard to measure, and show values ranging from a few to 50% of plasma insulin [202-204]. The interstitial insulin profile is important for the conclusions in Paper I, and the link should be further developed when reliable measurements of the adipose tissue interstitial insulin concentration in response to a meal are available.

**Figure 19: Modeling approach with dynamic constraints**

Dynamic constraints are used as additional experimental data in the estimation of parameters in hypothesis testing with mathematical models. Models must fit both *in vitro* data and dynamic constraints to be acceptable and not rejected. See also **Figure 2**.
Mathematical models linking insulin signaling to glucose control

There have been a few modeling projects dealing with the linking of intracellular insulin signaling models to whole-body models of glucose control (reviewed in [10, 12]). Chew et al. [205] have linked the Sedaghat insulin signaling model [142] with an early model for whole-body glucose homeostasis [206]. Also, in another effort the Sedaghat model has been linked to models for plasma insulin and glucagon concentrations, insulin and glucagon signaling, and glucose production and utilization [207, 208]. However, these modeling projects are using the Sedaghat model, and the Sedaghat model is based on non-human and non-dynamic data. In Paper I, we developed our own linking approach based on minimal models of insulin signaling and dynamic constraints from the Dalla Man model for glucose control [195].

In Paper I, we developed a link between intracellular insulin signaling models and a whole-body glucose control model. In the connection we used minimal insulin signaling models and dynamic constraints from the whole-body level. We also added more details in the insulin signaling model and even more detailed models as sub-models. Modified from Paper I.

In the minimal model analysis in Paper I, we concluded that at least two parameters had to be different between the in vitro and in vivo conditions in order for the intracellular model to account for the glucose uptake by the adipose tissue in the whole-body model. This conclusion would not have been possible without our two-step approach with an initial minimal model analysis (Figure 20). We examined two parameters that possibly are...
different between in vitro and in vivo conditions: handling of cells and blood flow. Handling of isolated adipocytes has been shown to enhance glucose uptake by the cells, i.e. the basal glucose uptake [209]. It is known that insulin increases the adipose tissue blood flow [210], and with higher blood flow, more glucose can reach and be taken up by cells. With these two differences in parameter values between the in vitro and in vivo conditions, we could fit all data both on the intracellular and the whole-body levels. We could thus continue to fill in the known details in the intracellular insulin signaling models. For instance, we tested to insert a detailed insulin binding sub-model [19] (Figure 20). The analysis in Paper I showed that it is possible to link detailed models from different levels using a minimal modeling approach and dynamic constraints. We used the results obtained in Paper I in the linking of the comprehensive insulin signaling model in Paper III with the Dalla Man model of glucose control [195].

With the results of Paper I and III we could also link the insulin resistance simulations in T2D to whole-body glucose control in T2D. This was possible since the Dalla Man model also exist in a T2D version, where glucose and insulin profiles during a meal correspond to the diabetic state [195, 211]. The Dalla Man T2D model was developed using the same equations as the normal Dalla Man model with re-parameterization of all parameters to limited data from T2D subjects. The linking between the Dalla Man T2D model and our developed insulin signaling model for T2D could not provide strong conclusions, but is nevertheless the first example of a multi-level model for insulin resistance in T2D. Simulations of the multi-level model for T2D demonstrated that an increased feedback from mTORC1 to IRS1 Ser 307 increases the dynamic adipose tissue glucose uptake in response to a meal (Paper III).
CONCLUDING REMARKS

In this thesis, I have focused on insulin signaling in human adipocytes; how these cells become resistant to insulin in type 2 diabetes, and how to connect such intracellular data with \textit{in vivo} data from the whole-body level. I also show the potential of mathematical modeling to correctly extract available information from experimental data.

In Paper I, we discover the requirement of additional mechanisms to be able to link intracellular insulin signaling models and data with a whole-body model for glucose control. We suggest that handling of cells and blood flow could be such mechanisms and, with these mechanisms added, we show a successful linking of the models at different levels. This successful linking provides a possibility to simulate clinical responses to intracellular perturbations in the insulin signaling network.

In Paper II, we conclude that internalization of the insulin receptor is important for the transient dynamic profile of phosphorylation of IR and IRS1 in rat adipocytes. We find two different explanations to a counter-intuitive, but apparent, reversed order of peaks in the phosphorylation dynamics using minimal mathematical models. The two found explanations are: i) different responding pools of IR combined with limiting concentration of IRS1, and ii) a negative feedback to IRS1.

In Paper III, we provide a systems understanding of insulin signaling and of insulin resistance. Our main conclusion is that the reduced sensitivity to insulin in adipocytes from T2D subjects can be explained with an attenuated feedback from the protein complex mTORC1 to IRS1. We also present for the first time a connection between a detailed intracellular insulin signaling model and an \textit{in vivo} glucose homeostasis model, where both models are developed using data from normal as well as T2D subjects.

In Paper IV, we build on to the systems understanding of insulin resistance with an inclusion of the ERK signaling branch in the detailed insulin signaling model. We use a minimal modeling approach to show that sequestration of inactive ERK is important for the slow dynamics of ERK phosphorylation, and for its return to basal levels. We also demonstrate a substantial crosstalk between ERK and mTORC1 signaling to control the ribosomal protein S6 and thus protein synthesis.
We claim to have achieved a first systems understanding of insulin resistance in human adipocytes. What do we mean by a systems understanding? The answer to that question concerns both the way we collect data and our mathematical modeling approach. In the data collection, we use the same cell type from the same laboratory to ensure that our data are compatible. We also obtain both steady state measurements with different insulin inputs, as well as dynamic data where we measure the response to insulin over a period of time. With both these kinds of data for several signaling intermediaries, we believe we capture the dynamic and input dependent behavior of the system. Finally, we obtain data from both non-diabetic and type 2 diabetic subjects – data that are directly comparable – to study the differences both in the magnitude of response and in the sensitivity to insulin for the signaling proteins. The systems understanding of insulin resistance occurs when we use all these data simultaneously in a mathematical modeling approach where the mechanisms of insulin resistance can be identified.

The way we use mathematical modeling has a few unique characteristics: parameter estimation, minimal modeling, and the use of core predictions. We estimate the parameters of the models, i.e. we do not rely on directly measured parameter values which have been tradition in systems biology. There are no reliable measurements of insulin signaling parameters in human adipocytes, but even if there were, these measurements come with an uncertainty that need to be taken into consideration – a fact that often has been neglected by researchers. To be able to estimate parameters, we need to work with as small models as possible, since the optimization problem grows with the square of the number of parameters. We thus use minimal models to find the important mechanisms and fill in more details at a later stage. These details, however, consists only of the things we actually measure, or mechanisms that are essential to explain the experimental data. Another issue with the unknown parameter values is the insufficiency of studying only the single best set of found parameters. Instead, all parameters that provide simulations of the model with statistically acceptable fit with data must be taken into account. The reason for this is that we want to obtain reliable predictions. Different parameters with similar fit with data can namely give totally different predictions for unmeasured variables. We thus need to study all the different parameters to be able to evaluate the uncertainty in the prediction. Predictions with low uncertainty, i.e. core predictions, are interesting predictions since they
can be used to reject or validate the model with the corresponding data. This way of using mathematical modeling we refer to as conclusive modeling since we perform our modeling analyses to be able to draw strong conclusions.

Why use mathematical modeling at all? Can the models provide us with new knowledge? How can we know that the models are correct? These questions address the skepticism that still exists when mathematical models are used in biological and medical research. First, a mathematical model is only “correct” for the data used in the development and testing of that particular model. Data for different cells and different experimental conditions can usually not be simulated with the same model without compensatory parameters. Second, a mathematical model is an approximation and always a simplification of the biological system of interest – which indeed is the very meaning of the concept “model”. In fact, our total knowledge of any biological system will always remain a simplification of the underlying layers of complexity. There will always be new layers of understanding to discover since research is a never-ending process. I believe that our search for answers improves with the use of mathematical models. The reason for improvement is that the use of mathematical modeling forces us to strictly formulate questions and hypotheses. We need to specify what we know and which assumptions we have to introduce. This process brings new light upon the existing data, and often guides us in what new experiments to perform, i.e. the experiments that give the most valuable contribution to the further understanding. A final reason to use mathematical modeling is that the approach provides a tool to simultaneously handle and analyze large data sets, and thus allow for a more correct and complete data interpretation. Unfortunately, the potential of mathematical modeling is yet far from fully realized within biological and medical research.

The mathematical modeling of intracellular insulin signaling and of insulin resistance in adipocytes is not completed with this thesis. For example, the mathematical models developed herein do not account for the important anti-lipolytic effect of insulin in adipocytes, i.e. that insulin counteracts the signaling to release of fatty acids. The anti-lipolytic signaling pathway of insulin and the crosstalk to insulin signaling for control of glucose uptake and protein synthesis may also include potential new mechanisms of insulin resistance. An interesting aspect of anti-lipolysis is that insulin directly interacts with β-adrenergic signaling. Another aspect of the lipolysis control is that fluxes of fatty acids to and from adipocytes contribute to the whole-body energy homeostasis. To be able to handle the effect of these fluxes, we have started a project to extend the whole-body model for glucose control [195] with models of glucose and fatty acid dynamics [212], and of insulin-regulated fatty acid dynamics in response to a meal [213]. A new version of the whole-body model will thus include glucose, insulin, and fatty acid dynamics.
The further development of the multi-level model should also include other insulin and glucose responding organs of importance for glucose homeostasis. Herein, we have contributed with a link to intracellular knowledge from adipocyte insulin signaling, but there are models of liver metabolism [214, 215], pancreatic insulin release [216, 217], and brain control of glucose homeostasis [218, 219], which could potentially further expand the presented multi-level model. Of great interest are, of course, also models of control of skeletal muscle insulin signaling and metabolism. Other future extensions to the multi-level model could include models for other perturbations like exercise [220] or longtime fasting. It would also be interesting to include disease progression of T2D into the model, which also has been described with mathematical models [221].
ACKNOWLEDGEMENTS

As long as my memory reaches, I have enjoyed mathematical problem formulations and equations. I am fascinated by the process of reformulation – from general questions to strict formulations with equations and logic relations where every assumption has to be clearly defined. During my time as a PhD student I have had the fortune to combine my passion for mathematics with interesting research questions within the field of insulin signaling, adipocytes, and diabetes. I have had a great time and I have learned a lot during these years. There are quite a few people who have contributed to this thesis, my situation at work, and life in general during these years. I would like to especially thank:

Peter Strålfors, thank you for your enthusiasm and presence in all my projects. You believe in me and are always supportive. I think we have developed a good work flow with our different competences and perspectives during these years.

Gunnar Cedersund, with positive and never ending energy you have boosted my projects. You are spreading a vital spirit that research is not the only important thing in life. I hope you will continue to believe in goodness of the humankind.

Cecilia Brännmark, you are extraordinary. We were always the best team and I hope we will start more joint research projects in the nearby future. Hopefully your mission as a spy at Astra Zeneca will provide me a postdoc position 😊

Meenu Rajan, you are a lab-machine. I enjoy collaborating with you since you are always in control of your work. You also broaden my perspective about life in general and India in particular.

Robert Palmér, you will go far with your ambitions. Our joint projects have been really successful and I hope for more collaboration projects in the future.

Siri Aili Fagerholm, you are a fighter. You have been involved in many of my projects, and collaborating with you is always a pleasure. Plan 12 will have to fill up an empty gap from you now that you leaved (after countless years).
Thank you my great group colleagues: Cecilia Jönsson for passionate leftwing debates in the coffee room and a special (not always understandable) sense of humor; Åsa Jufvas for keeping things in strict order at work, arranging Boda Borg competitions, and loud laughs in the corridors; Martin Öst for your readiness to help and taking care of practical issues at work; Rikard Johansson for your never ending jokes and games – at work and at our numerous joint trips to conferences (for example our crazy never-ending 1200 km road trip to Leipzig).

My excellent students: Linnéa Bergenholm, David Jullesson, and Eva-Maria Ekstrand, thank you for substantial contributions to my research projects and to a lively atmosphere at work and great moments at conferences we have attended together. I wish you good luck with your own PhD studies.

Thank you the remaining crew at Plan 12 for discussions about politics, society, life, and of course research in the coffee room. Anita Öst, your passion for science is inspiring. Mats Söderström, you are a reliable company at the morning coffee. Liza Alkhori, do you still think my research is easy? Shadi Jafari, you work hard (even without pressure) and I think we should play curling together in the near future. Asif Aziz, always well-mannered and polite with a perfect Swedish accent. Johanna Karlsson, your weekly visits lift the spirit at Plan 12. Daniel Nätt, I think we share many ideals in life. Annika Thorsell and Mattias Alenius with your research groups, Lovisa Holm and Susanne Hilke, you all contribute to the nice atmosphere for work and fun at Plan 12.

Thank you all other previous and present colleagues and collaborators at LiU, especially: Fredrik Nyström, Karin Lundengård, Mikael Forsgren, Maria Turkina, Björn Ingelsson, Olivia Forsberg, Sebastian Schultz, Tobias Strid, and Jordi Altimiras.

Thank you Stefan Klintström, Charlotte Immerstrand, and all other members of Forum Scientium for creating an inspiring multidisciplinary research environment with several social events and study trips around the world.

Thank you Fianne Sips and Natal van Riel at TU/e, Eindhoven, for fruitful collaborations throughout the years.

Jan Brugård and all employees at Wolfram MathCore, thank you for including me and keeping the contact during these years, also thank you for all the nice social activities I have been invited to.
I am also grateful to my amazing friends that have filled my life with meaning and pleasure during these years. Gävlesekten (undercover name: FGF) – my best company for outdoor activities and adventurous travels. I hope (and think) you will never grow up and become boring. Luciatåget, we have been singing, cooking, and partying together throughout these years, but unfortunately it is harder and harder to find time to hang out. Previous and present members of Matlaget provide me with nicely cooked vegan meals with folköl every week combined with conversations covering the important issues in life. Thank you also Vuxengänget for study motivation the years before I got my PhD position and remaining friendship throughout the years. Carin Engsjö, I miss your bubbling energy around me. Rome and Linköping are at least 2000 km too far apart.

Storasyster Maria, you have been an inspiration for me to continue studying, but now I am far ahead of you in filling my brain with knowledge. You build and create with impressing energy. Good luck with the licorice, and your growing cottage and family.

Lillebror Lars, always calm and stable. It is perfect to have a real computer expert in the family, especially since I have had the role as the computer nerd in my research group.

Lillasyster Stina, organized, positive, and always on full speed. It is great that you have moved to Linköping so we can play volleyball, board games, and work for a fairer world together.

Mamma Gertrud and Pappa Bengt, thank you for the opportunity to make my own choices in life. You have supported me both when I travelled to different corners of the world and during endless years of studies – even though you never understood my math assignments 😊

Anders, we have so much fun together, both in the everyday life and during vacations. I cannot imagine a better life companion. Thank you for support and presence during these years. You will always be close to me.
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Papers

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