Chapter 3

Integration of transcriptional data into ADAPT improves its estimation on changes in lipid metabolism induced by liver X receptor activation

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ABSTRACT

Pharmacological interventions induce progressive metabolic adaptations. Understanding the molecular basis of metabolic changes is complicated by the timescale at which the changes occur and by multilevel regulatory mechanisms in the affected metabolic pathways. In chapter 2 we described how a modeling approach called ADAPT (Analysis of Dynamic Adaptations in Parameter Trajectories) was used to predict the progressive alterations in and origin of hepatic steatosis and hypertriglyceridemia upon treatment with the liver X receptor (LXR) agonist T0901317.

In this chapter we show that inclusion of information on gene expression data into this mathematical model of LXR agonism improved its predictive capability. While inclusion of gene expression data reduced the variety in many of the model's predictions, not all parameters could be effectively constrained. We highlight how inclusion of transcriptional data made it possible to model the subcellular fractionation of accumulated hepatic triglyceride (TG) induced by LXR agonism. This subcellular fractionation was subsequently validated by an independent in vivo experiment.

In conclusion, addition of information about the transcriptome in computational modeling can be valuable in making quantitative predictions about the multilevel regulation in complex metabolic systems.
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INTRODUCTION

Because of their positive impact on cholesterol metabolism [71, 72, 73, 74] and potent anti-atherosclerotic effects [75] liver X receptors (LXRs) are considered attractive drug targets [76, 77]. LXR agonists however also have detrimental side-effects, causing severe hepatic triglyceride (TG) accumulation and hypertriglyceridemia in animal models [71, 72].

The study of metabolic adaptations to pharmacological interventions such as LXR agonism is complicated by the timescale at which the changes occur: the effects on plasma TG are transient [71, 78, 93, 94, 95, 130] and the increment in hepatic steatosis is time-dependent [130]. A further complication in understanding the molecular basis of metabolic changes is posed by multilevel regulatory mechanisms. While there is a wealth of information available on changes in the transcriptome level, it is not known how and under which conditions metabolic fluxes are altered by interaction with the transcriptome or proteome levels [131].

Systems biology aims to use mathematical modeling of metabolic systems to improve the understanding of complex biochemical networks. These computational models are simplifications of complex biological networks and can be used to gain mechanistic insight into the biological system. A mathematical model describes states which typically represent metabolite concentrations. These states are interconnected via fluxes, representing the transport or conversion of metabolites. The mathematical equations of the fluxes contain parameters that determine the kinetics and dynamics of the biological system. Classical modeling approaches assume that these parameters are constant in time. However, for disease processes and pharmacological interventions, this is typically not the case.

In chapter 2 we described how a mathematical approach called ADAPT (Analysis of Dynamic Adaptations in Parameter Trajectories) was used to predict the origin of hepatic steatosis and hypertriglyceridemia after different time periods of treatment with the LXR agonist T0901317 [130]. For this purpose the model was extended using hepatic gene expression data. This chapter shows how the inclusion of information on hepatic gene expression data improved the predictive value of this mathematical model of LXR agonism.
EXPERIMENTAL PROCEDURES

Animals and experimental design
All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Groningen. Animal experiments and analyses on animal tissues for development (and validation) of ADAPT were carried out as described before ([91, 92, 130], see also chapter 2). Briefly, male C57Bl/6J mice (Charles River, L’Arbresle Cedex, France) were housed in a light and temperature-controlled facility and fed a standard laboratory chow diet (RMH-B, Abdiets, Woerden, The Netherlands) containing T0901317 (Sigma-Aldrich, St. Louis, MO, USA; 0.015% wt/wt; 30 mg/kg) for the indicated time intervals. Groups of untreated C57BL/6J mice, receiving non-supplemented laboratory chow served as controls. After a 4 hour fast (8-12 AM), animals were sacrificed by cardiac puncture under isoflurane anesthesia. Livers were quickly removed and either freeze-clamped and stored at -80°C or immediately used for further analysis.

Hepatic gene expression analysis
RNA was extracted from livers using Tri Reagent (Sigma-Aldrich, St. Louis, MO, USA). cDNA obtained by reverse transcription was amplified using the appropriate primers and probes. Primer and probe sequences are listed in Appendix A. All mRNA levels were calculated using a calibration curve, expressed relative to the expression of β-actin and normalized to expression levels in untreated mice.

Integration of gene expression data into ADAPT
The version of ADAPT that was used in this study describes how 11 metabolites (x1-x11) are interlinked by 29 fluxes (f1-f29). The equations that describe the fluxes (Appendix B) contain parameters that determine the kinetics and dynamics of the biological system. ADAPT uses a time-dependent evolution of model parameters to predict how the modeled metabolite concentrations and fluxes change over time. For a detailed description of the modeled metabolites, flux equations, and parameters the reader is referred to Appendix B.

ADAPT was extended using integration of hepatic gene expression data concerning TG metabolism (for details see Appendix B). Time-course data of relative gene expression levels were used to constrain the dynamics of parameter trajectories. For this purpose, mRNA expression levels of Ldlr, Vldlr, Lrp1, Cd36, Fabp4 (Ap2), Mttp, Apob, Lpl, Acadl (Lcad), Acox1 (Aox), Hmgcs (Hmg-
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coaS, Ucp2, Gpat, Fasn (Fas), Me1, Srebpf1 (Srebp-1c), Scd1, Abcg1, Abcg5, Cyp7a1, Fdft1 (Sqs), Hmgcr (HmgcoaR), and Srebpf2 (Srebp-2) were included in the computational analysis. In Table B.3 an overview of the gene-parameter couples and associated fluxes is provided.

Pathways at the transcriptome level cannot be modeled explicitly due to the lack of sufficient quantitative information about this system. Therefore, a different approach is required to integrate gene expression data in the parameter trajectory estimation. The approach used here is based on the assumption that changes in metabolic parameters are reflected by changes in corresponding enzymes, which in turn are reflected by changes in corresponding gene expression levels. Parameter estimations were adapted in such a way that when parameter trajectories and gene expression profiles positively correlated, they were preferred over scenarios where they did not. For this purpose an objective function was included that maximizes the temporal correlation (Pearson correlation coefficient) between parameter trajectories and gene expression profiles in parameter estimations. Gene expression data was also used to constrain the magnitude of dynamic variations in the parameter trajectories. It was assumed that parameters are less likely to change when corresponding gene expression levels do not change, compared to when gene expression is induced or repressed. An extra objective function that utilizes the time derivative of gene expression profiles to constrain parameter fluctuations was included. Parameter trajectories were estimated using 200 time steps. A collection of 20000 parameter trajectory sets was obtained that describe the experimental data. For the ADAPT analyses presented in this manuscript, we used 1000 parameter trajectory sets that displayed the highest temporal correlation with gene expression profiles.

Hepatic subcellular fractionation
Livers were fractionated into cytoplasmic and microsomal membrane fractions essentially as described [132]. Briefly, freshly excised livers were homogenized (10% w/v) in ice-cold buffer (0.25 M sucrose, 3 mM Tris and 1 mM EGTA, pH 7.4). Homogenates were spun at low speed (9000 xg for 10 min) to remove cellular debris followed by high speed centrifugation (105000 xg for 2 hours) to separate the cytosomal lipid fraction from the intact microsomal membrane fraction. TG content of the collected fractions was determined using a commercially available kit (Roche Diagnostics, Basel, Switzerland).
RESULTS

Adding transcriptional data to ADAPT reduces the variance in predictions over time

Including gene expressions leads to reduction in prediction variability in many of the components of the model, which is shown by a (dark) gray colour in Figure 3.1. Also parameters that are not coupled to gene expression data show reduction in variability (Figure 3.1). When more closely examining the metabolic states ($x_1$-$x_{11}$, Figure 3.1, left column), it is noteworthy that mainly predictions on hepatic metabolites ($x_2$-$x_7$), but not plasma metabolites ($x_8$-$x_{11}$), were effectively constrained by adding gene expression data. This is presumably because hepatic gene expression data was coupled to parameters that describe fluxes in hepatic lipid metabolism, which are more closely linked to hepatic metabolite concentrations, than to plasma metabolite concentrations. Interestingly, parameters and fluxes describing hepatic de novo TG synthesis ($p_7$, $p_{10}$, $f_7$ and $f_{10}$) were not effectively constrained by inclusion of transcriptional data (Figure 3.1, middle and right column), while these parameters are coupled to gene expression data. This is in accordance with our data in chapters 2 and 4 that show that hepatic lipogenic gene expression does not accurately predict de novo fatty acid synthesis [130].

Integration of transcriptome into ADAPT predicts subcellular distribution of hepatic TG

The gradual increase in hepatic TG induced by T0901317 treatment can be accurately simulated using ADAPT, either with or without using gene expression data (Figure 3.2, upper panels). Hepatic TG can either reside in lipid droplets in the cytosol or may be incorporated into very low density lipoprotein (VLDL) for secretion into the circulation. TG is integrated into VLDL in the endoplasmic reticulum (ER) (reviewed in: [133]). Before including information on gene expression data, ADAPT did not predict how hepatic TG content was distributed over the cytosol and ER (Figure 3.2). However, when including gene expression data, model predictions show that the increased TG fluxes upon T0901317 treatment would especially be stored in the cytoplasm, rather than in the ER (Figure 3.2).

In vivo data validate the predicted subcellular distribution of TG

To validate the prediction on subcellular distribution of hepatic TG, we performed fractionation of TG in livers of mice treated with T0901317 for 14 days
Inclusion of gene expression data reduces variability of many model predictions. Gray areas indicate effective temporal variance reduction (on a scale from 0 = no reduction to 1 = most efficient reduction) for a) metabolites (states), b) parameters and c) fluxes. A detailed description of the modeled states, fluxes and parameters is given in Appendix B. *indicates which parameters were coupled to gene expression data (see also Table B2.3).

and untreated mice. Indeed, as predicted using ADAPT, the bulk of TG was found in the cytosolic fraction of liver tissue in T0901317 treated as well as in untreated mice. Also, an increase in TG concentration was observed in the microsomal (ER) fraction upon T0901317 treatment (Figure 3.3).

DISCUSSION

The underlying cause and progression of the multilevel effects of LXR agonism are not well understood. Computational modeling strategies can be used to gain mechanistic insight into gradually changing adaptive processes resulting from pharmacological interventions, such as LXR activation. The metabolic changes that occur as a consequence of treatment likely result from a complex
Figure 3.2: ADAPT predicts that hepatic TG fluxes are increased and preferably stored in cytoplasmic fractions upon T0901317 treatment. Comparison of simulations of TG compartmentalization in cytoplasm and ER with and without gene expression modeling. Trajectories of total hepatic TG content ($x_4 + x_5 + x_6 + x_7$), as well as its subdivision into cytoplasmic ($x_4 + x_6$) and ER ($x_5 + x_7$) fractions are shown. Gray areas represent 95% intervals. In the upper graphs in vivo experimental data on hepatic TG are represented as lines ranging from upper to lower standard deviation.

interplay of many components of the affected biological network. Thus, to understand the progression of multilevel effects of LXR agonism, molecular data of the transcriptome, proteome and metabolome should be integrated. In this chapter we show that ADAPT was successfully extended by integration of transcriptional data, improving its predictive value.

When constraining parameters in ADAPT with time-course data of gene expression levels, it was assumed that changes in metabolic parameters are reflected by changes in corresponding enzymes, which in turn are reflected by changes in corresponding gene expression levels. This implies that there is a functional relationship between a metabolic parameter and corresponding gene expression level. It is however not known to what extent transcriptional data
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**Figure 3.3:** *In vivo* experiments validate the predicted subcellular TG division upon T0901317 treatment. Subcellular division of hepatic TG in the cytosol (1) and ER (2) in C57Bl/6J mice treated with T0901317 for 14 days and untreated controls. Values are depicted as boxplots for n=6 mice per group (middle line: median, box: 25th to 75th percentiles, whiskers: 5th to 95th percentiles), *p*<0.05 vs. untreated controls (Mann Whitney U).

can be indicative for changes in metabolic pathways, because of the complex multilevel organization of metabolism [131]. Correlation of gene expression data to protein levels [134, 135, 136, 137, 138], enzyme activities and fluxes [139, 140] through metabolic pathways varies widely. In our experience for instance, lipogenic gene induction in the liver does not predict increased *de novo* synthesis of fatty acids under all circumstances (chapters 2 and 4). Lately however, several studies have shown the value of integrating gene expression data in computational techniques, e.g. [139, 141, 142, 143, 144]. Importantly, ADAPT does not assume a direct relation between gene expression and metabolic activity. The method uses information in the gene expression data to reduce variation in predicted concentrations and fluxes as long as this does not violate the description of the metabolic data.

Our data show that inclusion of information about the transcriptome effectively constrains the variation in predictions in many components of the model. Interestingly, in some cases predictive value of parameters and fluxes that are not coupled to gene expression data was improved, while in other cases no improvement was found in parameters and fluxes that are coupled to gene expression data. In particular, information about lipogenic gene expression did not improve the predictive value on *de novo* synthesis of TG. The observation that hepatic expression of genes involved in *de novo* fatty acid synthesis does
not always predict flux through the lipogenic metabolic pathway was also made in another animal model (chapter 4). The difference in effectiveness of the constraints may be regarded as a reflection of the complex multilevel regulation of metabolism [131].

Hepatic TG can be stored inside lipid droplets in the cytosol or in VLDL in the ER. Incorporation into VLDL is facilitated by microsomal triglyceride transfer protein (MTTP) (reviewed in: [133]). Inclusion of transcriptional information into ADAPT (also hepatic Mttp expression), led to a reduced variation in predictions on the subcellular fractionation of hepatic TG. Independent experimental data validate this prediction on subcellular distribution of hepatic TG upon LXR activation. In livers of mice treated for 14 days with T0901317 we observed that TG were preferably stored in the cytoplasm.

In summary, while gene expression data do not always predict the flux through a metabolic pathway, including information on this regulatory level of metabolism can improve the predictive value of ADAPT. Metabolism is regulated by a complex interplay of factors, from genes and proteins to metabolites, post-translational modifications, hormones and inter-organ communication. Including data about these other levels of regulation may further improve the predictive value of computational techniques and may help to gain insight into regulatory pathways.

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