

## Modulation of Metabolism Through Transcriptional Control has created New Treatment Opportunities for Type 2 Diabetes

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**Abstract:** The discovery of the important metabolic and physiological role played by a family of transcription factors, the peroxisome proliferator activated receptors (PPAR), has opened up for a new understanding of the mode of action for the lipid lowering drugs known as fibrates and for the new glucose lowering compounds described as insulin sensitizers.

Both of these classes of compounds have demonstrated significant efficacy in both animal models of the metabolic derangements characteristic for type 2 diabetes and in human clinical studies. The recognition of the role of these drugs as ligands for PPAR transcription factors and the development of new molecular and cellular tools to select and characterise new PPAR selective compounds will open up for the development of even better new drug candidates for the treatment of metabolic disorders associated with type 2 diabetes. With the combined strength of new transcriptional mapping technologies developed in the field of molecular biology, such as differential mRNA display and DNA microarray hybridisations, it will be possible to perform a detailed molecular characterisation of the transcriptional events involved in drug actions in cellular and tissue systems, and information gathered from such types of analysis will lead to an enormous amount of data, from which detailed knowledge of drug actions at the gene regulatory level will emerge.



### METABOLIC DISTURBANCES IN TYPE 2 DIABETIC PATIENTS

Although the etiology of type 2 diabetes is not known, several analyses suggest that the disease is the result of a combination of genetic susceptibility and external factors of which an increase in calorie consumption, especially fat, is by far the most important element. The impact of excessive calorie consumption on the development of the type 2 diabetes forms the basis for the almost epidemic increase in disease prevalence, which is seen throughout most of the world.

The hyperglycemia of type 2 diabetes is caused by a combination of impaired glucose uptake in muscle and fat tissue [1,2], increased hepatic glucose production [3] and finally a defect in glucose mediated insulin release [4,5]. The elevated level of plasma glucose is, however, only part of a more general dysregulation of "fuel utilization" such as metabolism of glucose and lipids in these patients [6,7]. The disturbances in lipid metabolism are profound and include elevation of plasma free fatty acids (FFA). The

increase in plasma FFA is probably a reflection of the impaired anti-lipolytic activity of insulin in fat tissue and of the increased amounts of fat tissue in type 2 diabetic patients [6]. Analyses suggest that elevated levels of plasma FFA can induce almost all of the changes in glucose metabolism seen in the patients. First, increased concentration of plasma FFA induces a state of insulin resistance in muscle [8] and other tissues. Second, clinical studies suggest that it is serum FFA and not insulin that primarily determines the glucose production from the liver [9].

In addition to having effects on glucose metabolism, the increased supply of FFA and glucose to the liver contributes to the overproduction of plasma triglyceride rich VLDL particles [10]. Adding to this overproduction of lipoproteins from the liver is a decreased activity of lipoprotein lipase [11], resulting in a prolonged circulation time of triglyceride rich lipoproteins. This forms the basis for the development of the diabetic dyslipidemic lipoprotein profile, characterized by elevated concentration of small, dense LDL particles, increased concentration of small, cholesterol-rich remnant particles, and decreased concentration of HDL cholesterol [12].

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Myocardial infarction, ischemic heart disease, and stroke are some of the macrovascular complications responsible for 80% of the mortality in patients with type 2 diabetes. Type 2 diabetics have a 3 to 5 fold increase in morbidity and mortality of coronary heart disease (CHD) compared to the normal population [13], and macrovascular complications have a much greater impact on patient mortality than microvascular complications. Recent clinical data suggests that small, dense LDL and low HDL cholesterol are very important factors in the development of macrovascular diseases. In fact, the risk factor posed upon the patients by the special diabetic dyslipidemic phenotype, far exceeds more traditional risk factors like elevated LDL cholesterol [14,15].

## **TRANSCRIPTION FACTOR LIGANDS AS MODULATORS OF LIPID METABOLISM AND PLASMA LIPOPROTEINS**

### **Gene Regulation of Lipid and Fatty Acid Oxidation**

Lipid lowering fibrates, of which many have been used in the clinic, are classified as peroxisome proliferators because they cause proliferation of peroxisomes. This proliferation is associated with hepatomegaly and hepatocellular carcinomas in rodents, phenomena that so far have not been reported in man. At the physiological level, an increased number of peroxisomes in the liver increase the capacity of this organ to perform oxidation of fatty acids, and fibrates thus cause a general reduction in the load of lipids and fatty acids in the body.

At the molecular level, the fibrates along with other groups of compounds such as certain leukotrienes, eicosanoids, and long chain fatty acids have been identified as ligands [16-18] for a recently discovered class of transcription factors described as peroxisome proliferator activated receptors (PPARs) of the  $\alpha$  subclass. PPAR  $\alpha$  is primarily expressed in liver, kidney and heart, where it through heterodimerisation with another family of nuclear transcription factors, the retinoid X receptors, RXRs, induces the transcription of mRNAs encoding enzymes like, e.g. acyl-CoA oxidase [19], bifunctional enzyme (enoyl-CoA hydratase/3-hydroxy-acyl-CoA dehydrogenase [20], lipoprotein lipase [21], malic enzyme [22] and medium chain acyl-CoA dehydrogenase [23], which are involved in the metabolism and oxidation of lipids and fatty acids. Importantly,

disruption of the ligand-binding domain of PPAR by homologous recombination in mice resulted in animals that did not display the peroxisome proliferator pleiotropic response when exposed to these compounds. After treatment of PPAR knock-out mice with peroxisome proliferators no hepatomegaly, peroxisome proliferation, and transcriptional activation of target genes were observed [24].

Fibrate mediated activation of PPAR  $\alpha$  also results in transcriptional induction of the major HDL apolipoproteins, apoA-I and apoA-II, which causes an increased HDL cholesterol. PPAR activation can decrease plasma triglycerides through a decreased hepatic apoC-III production and through an increase in LPL-mediated lipolysis. Thus, fibrates stimulate cellular fatty acid uptake, conversion to acyl-CoA derivatives, and catabolism by the beta-oxidation pathways, which combined with a reduction in fatty acid and triglyceride synthesis, results in a decrease in VLDL production [25]. In addition to inhibition of the VLDL production, inhibition of cholesterol transfer from HDL to VLDL results in normalization of the transformation of VLDL precursors to receptor-active LDL, thereby reducing the atherogenic small dense LDL particles [26-28].

The accelerated atherosclerosis, that is the major morbidity factor for type 2 patients, is associated with a lipid profile characterised by low HDL cholesterol, increased plasma triglycerides, and increased level of small, dense LDL. It is, therefore, tempting to speculate that fibrates may be valuable in preventing cardiovascular diseases in these patients. Most fibrates, in use as treatment for dyslipidemia, preferentially activate PPAR  $\alpha$  and recently, a positive correlation between PPAR  $\alpha$  EC<sub>50</sub> in transient transactivation assays and *in vivo* MED<sub>40-60</sub> (producing 40 – 60 % lowering of serum VLDL+LDL cholesterol) was reported [29].

In the Helsinki Heart Study [30] the effect of gemfibrozil on risk of coronary heart disease was evaluated in patients with elevated levels of non-HDL cholesterol. The study has not had major clinical impact, in that a 34% reduction of cardiac events for some reason did not translate into a reduction of mortality. Subsequent subgroup analysis has demonstrated, however, that almost all of the beneficial effect was seen in the population with a combination of low HDL-cholesterol and high triglycerides. Obese patients with this lipid profile had a cardiac risk reduction

of 75 % [31]. These data and the advent of more efficacious fibrates gives hope to ongoing intervention trials like the Diabetes Atherosclerosis Intervention Study [32] and the Fenofibrate Intervention and Event Lowering in Diabetes Study [33], in which dyslipidemic type 2 patients are treated with micronized fenofibrate.

### Gene Regulation of Fat Storage and Adipocyte Differentiation

Chemical synthesis of fibrate derivatives lead the way to the establishment of yet another new group of pharmacologically versatile compounds, the thiazolidinediones, which in animal models of type 2 diabetes and in the clinic have shown beneficial effects primarily as hypoglycemic agents and therefore have become known as insulin sensitizers. Early members of this class include Pioglitazone [34,35], Troglitazone [36,37] and Rosiglitazone [38-40], which are now available in or entering the diabetes markets in many countries including the USA and Europe.

In 1995 Lehmann *et al.* discovered that potent insulin sensitizers acted as high affinity ligands for PPAR [41]. This finding naturally led to the idea that the hypoglycaemic properties of the insulin sensitizers were mediated by this orphan nuclear receptor. This has subsequently been substantiated by the finding of a positive correlation between potency of PPAR activation in transient trans-activation assays ( $EC_{50}$ , see below) and the minimum effective dose needed to attain 25 % maximum hypoglycaemic effect ( $MED_{25}$ ) in ob/ob mice [35].

In contrast to PPAR, PPAR is primarily responsible for the transcriptional regulation of genes involved in adipocyte differentiation and at the metabolic level in FFA and lipid anabolism and storage. Examples of such target genes include acyl-CoA synthase [42], adipocyte lipid binding protein (aP2) [43], phosphoenolpyruvate carboxykinase [44], brown adipocyte uncoupling protein [45] and stearoyl-CoA desaturase 1 [46].

The PPAR mediated reduction of hyperglycemia observed in type 2 diabetes is speculated to be secondary to adipocyte differentiation [47] or activation [42,48]. Metabolically active adipocytes will presumably store more lipids and thus decrease circulating levels of free fatty acids, and upon treatment with insulin sensitizers the body can gradually base more of its energy consumption on the oxidation

of glucose in insulin responsive tissues such as muscle. While one might expect an increased body weight from such treatment, this has apparently so far not been a major problem, but future long term treatment of patients will answer this question.

### Characterisation of New PPAR Ligands in *in-vitro* Assays

Over the past decade, a number of technologies have been applied to describe the interaction between ligands and PPAR receptors. These interactions have been characterized essentially at three levels: First, the interaction has been described in terms of direct binding using classical and novel type binding assays yielding  $K_d$  values as measure of the interaction. Second, interactions have also been characterized by the use of functional assays in which the transcriptional activity of the ligand stimulated receptors have been determined either on native promoters containing peroxisome proliferator response elements (PPRE's) or from heterogeneous promoters as e.g. Gal4 fusion proteins. This type of assay yields both a measure of the efficacy and the potency of a compound. Third, drug interactions with PPAR and PPAR have also been investigated in spatial terms through the co-crystallization of PPARs with their ligand.

The first step in the identification of a new ligand is often the use of a ligand-binding assay to directly detect binding to the receptor in question [18,49-52]. Classically, this involves the use of a radio labeled ligand and recombinant PPAR protein typically expressed in *E. coli*. Displacement of the receptor bound radio labelled ligand allows determination of  $K_i$  for the compound in question. This technology can be formatted for high throughput screening in the form of an SPA (scintillation proximity assay) based assay [53]. In addition, a number of alternative techniques are also available to detect and quantify direct binding. These include the protease sensitivity assay, ligand-induced complex formation assay (LIC) and co-activator-dependent receptor ligand assay (CARLA) [54]. These assays take advantage of the conformational change induced in the receptors upon binding to ligand. The protease sensitivity assay is based on the observation that when the receptor is exposed to protease a protected fragment that is not observed with receptor alone emerges from the ligand-receptor complex. The LIC assay was developed using band shift assay to detect PPAR-receptor-RXR interaction [16], whereas the CARLA assay

detects ligand dependent interaction of the receptor with a co-activator protein or a fragment hereof [55].

Ligands for the PPARs can also be identified and characterized in cell based assays measuring the ligand dependent transcriptional capacity of the receptors [41,29,52]. In these assays a reporter gene under PPAR transcriptional control is measured after addition of compound to the medium of transiently transfected cells. The reporter gene, may be controlled by a native PPRE, in which case the full-length receptor is used in the assay. Alternatively, a heterogeneous enhancer/ promoter such as the yeast GAL4 response element can control the reporter gene expression. In this setting the ligand-binding domain (LBD) of the PPAR that contains the ligand binding pocket and the ligand responsive activating function is fused to the DNA binding domain (DBD) of the Gal4 protein thus allowing for PPAR controlled reporter gene transcription. The full-length receptor approach most likely reflects the *in vivo* situation more closely than the fusion protein approach. However, since these assays are frequently carried out in cells from higher eukaryotes, the latter assay is likely to be less obscured by activities of endogenous factors.

The crystal structures of the human PPAR and LBDs in the presence or absence of ligand have been solved. The LBD consist of 13  $\alpha$ -helices and a small  $\beta$  sheet forming a hydrophobic ligand-binding cavity. Although the binding pockets of the two receptors are similar to that of other known nuclear receptors two major differences have been observed. Firstly, the binding pocket of the PPARs is about twice the size of that for other receptors. Moreover, only 30 and 40 % of the volume in the cavity of PPAR and binding pockets is occupied by rosiglitazone and eicosapentaenoic acid respectively [56,57]. This is unusual, since for instance the thyroid hormone receptor ligand occupies 90 % of the volume in the binding pocket. This raises the question of whether other larger ligands remain to be identified. Secondly, the PPAR LBDs contain an additional  $\alpha$ -helix at the bottom of the ligand-binding pocket that takes part in the entry into the pocket of the ligand. Understanding the spatial features of the ligand binding pockets of these receptors will be of great significance for the future development of novel improved insulin sensitizers and / or lipid lowering agents.

## Characterisation of New PPAR Ligands in *in vivo* Animal Models

Characterisation of PPAR ligands can be done in a variety of animal models. The db/db mouse is a convenient model for evaluation of the potency of larger series of PPAR compounds that will lower plasma glucose, insulin and serum triglycerides [58]. It is far more difficult to establish rodent animal models that in a reproducible way reflect the lipid lowering properties of PPAR ligands. Cholesterol lowering in cholesterol fed rats [59] or triglyceride lowering in hamsters fed a high fat diet (in-house observations) are some of the current alternative possibilities. Transgenic animal models like the human apoA-1 gene transgenic mice [60] or the apoE3-Leiden mice [61] may prove to be very useful.

## TRANSCRIPTIONAL MAPPING OF EXPRESSED GENES AS A TOOL IN THE IDENTIFICATION OF NEW POTENTIAL DRUG TARGETS

In many diseases, it is still not known which genes or gene products are involved in the pathobiology in question, but gene-technology now offers new approaches in the search for new candidate genes encoding putative pharmaceutical targets for drug discovery. In any differentiated cell type only a subset of all available genes is active to specify the particular functions characteristic for this cell type. However, disease conditions can modify the activity or expression levels of many important genes leading in some cases to changes that will have a direct causative relation to a particular disease state.

In the case of type 2 diabetes a selected number of genetic defects such as defects in the genes encoding the insulin receptor, glucokinase, and hepatic nuclear transcription factors lead to more narrowly defined subtypes of the disease. However, there are no strong indications that the common adult-onset type of diabetes should have as its primary cause a defect at one or a few specific genetic loci [62-65]. Nevertheless, a better understanding of the changes in the regulation of gene transcriptional activity, which is associated with the development of metabolic dysregulation, might lead the way to identification of new treatment modalities.

## **Transcriptional Mapping of Genes Regulated in Disease or upon Treatment with Drugs**

With our current stage of knowledge, where only a fraction of the human genes have been identified and functionally described in detail, we do not always know all the genes that are involved or modulated by disease. This is even the case in many conditions, where there is a significant genetic component involved in the etiology of the disease. However, we can now systematically investigate if transcriptional up or down regulation of genes in afflicted cells and tissues may be associated with the pathological situation, and we may even be able to identify novel and important genes, which may form the basis for pharmaceutical intervention.

This methodology is called transcriptional mapping of gene regulation, and is currently mostly based on work conducted on cell culture derived material or on tissue material from appropriate animal models, which may serve as simplified surrogates for the human diseases under study. However, there is in principle no reasons why such studies could not be conducted on tissue material collected from e.g., patients enrolled in clinical studies, and we will presumably soon see various applications of transcriptional mapping used in extensive studies in man.

In transcriptional mapping, all genes, irrespective of whether their exact identity or function is known or not, can be compared between tissues collected from patients and from control individuals. Such comparisons can lead to identification of defects in the gene regulatory machinery, which might be causative for specific disease conditions. This knowledge will have the potential to lead to new opportunities for pharmaceutical development.

A detailed characterisation of the mode of action at the molecular level of investigational compounds and drugs that act at the level of gene regulation can also be obtained through transcriptional mapping. In such studies the mapping is performed on cells or on tissue material from animals treated with the compounds in question. By administering ligands for PPAR and PPAR to responsive cell cultures or to animal model systems, it is thus possible to obtain a detailed picture of overall gene regulation caused by the ligands for these transcription factors.

## **Differential mRNA Display Analysis of Gene Regulation**

Differential mRNA display analysis, which is one well-established way of performing transcriptional mapping, can be used to investigate the overall gene regulatory patterns in a biological sample. Differential mRNA display analysis was devised and refined by Liang and Pardee [66] as a method to compare gene expression in two or more cell types and to clone differentially expressed genes rapidly. The method is based upon a series of steps; reverse transcription of mRNA from anchored primers, rounds of polymerase chain reactions (PCR) using the same anchoring primer and a short arbitrary primer as well as radioactive nucleotides, and finally labelled cDNAs produced by PCR are separated on a DNA sequencing gel and visualized by autoradiography.

## **DNA Array/Chip Analysis of Gene Regulation**

As an alternative to the overall analysis, in which no prior knowledge of the identity or DNA sequence of the genes investigated is required, it is now also possible to immobilise thousands of gene fragments on solid supports either as cDNA fragments [67-69] or as oligonucleotide counterparts [70]. Such immobilised DNA probes can be hybridised with dye-labelled cDNA mixtures, which have been reverse transcribed from mRNA populations isolated from cells and tissues under investigation. The resulting hybridisation patterns will give detailed information on the regulation at the transcriptional level of all of the pre-selected genes included in the immobilised set of probes.

New highly automated technologies in this field have required significant resource investments, both in terms of development of the necessary hardware needed for robotization of the handling of gene fragments to be analysed, and in the development of the software for final analysis of accumulated data.

Equipped with sufficient and appropriate access to databases with a rapidly accumulating number of mammalian and human gene sequences combined with access to the relevant biological samples from adipocyte, muscle and hepatocyte cell cultures and from animal models of dyslipidemia, the power of these new analytical technologies is now explored, and the defined aim is to obtain a better understanding at the level of

gene transcriptional regulation of the development of metabolic dysregulation seen in type 2 diabetes.

### New Target Identification and Evaluation

It is expected that this technology will provide the scientific community with a better understanding of the genetic circuits involved in metabolic regulation as well as create new possibilities for identification of molecular targets for therapeutic intervention. Once such candidate gene targets have been discovered by transcriptional mapping, the pharmaceutical industry still has a long path to travel before the specific biology of the newly discovered genes is unravelled and before new drug candidates acting on these genes and their protein products can be identified.

Gene target evaluation will involve at least over- and underexpression of the selected full length genes initially in *in vitro* systems and possibly also in tissue specific and in development-stage specific *in vivo* systems such as transgenic and gene knock-out mouse models followed by careful biological assessment of the physiological and metabolic consequences of such changes.

In this respect it is important to realise that any drug that affects transcriptional regulation has the potential to modulate promoter activities at genetic loci that may be involved in other unrelated cellular functions. Such functions, if they are centrally involved in regulation of cell growth and differentiation, may represent targets that it would be potentially hazardous to modulate in a non-physiological direction.

Therefore, since the PPAR transcription factors are centrally involved in cell differentiation in several tissues, transcriptional mapping technologies also hold the potential to identify and characterise the regulation of genetic elements that not only regulate metabolic functions, but also those that are of particular relevance for coordinated growth and differentiation of other cell types.

Of particular interest is the involvement of PPAR in the development of the epithelium in colon. PPAR ligands have been shown to have potent antineoplastic and antiproliferative activities both *in vitro* [71,72] and *in vivo* as judged by growth inhibition of human colonic tumours transplanted in to mice [73]. These

findings, however, contrast results obtained in rodent models characterised by spontaneous development of multiple intestinal tumours due to a mutation in the adenomatous polyposis coli (APC) tumour suppressor gene. Treatment with PPAR ligands was found to promote development of colonic tumours in these animals, whereas no effect could be observed in wild-type animals [74,75].

### THERE WILL BE MUCH MORE TO BE LEARNED

An important interplay between differentiation biology and gene regulation has been taken for granted for many years, but the new recognition of the strong relation between transcriptional regulation, cell differentiation and metabolic regulation in common diseases like diabetes has added an interesting twist to the synergy between molecular biology and drug discovery and development. We have only begun to acquire a very superficial view and initial basic understanding of how this new field of research will help us in designing new treatment modalities based on transcription factor ligands. This will in the future allow us to investigate at the molecular level the associated efficacy and safety aspects related to the development of such drugs.

In addition to the already described classes of PPAR ligands new and interesting compounds for treatment of type 2 diabetes will be discovered and developed in the near future, and we feel that transcription biology offers a unique opportunity for providing access to potentially important new targets in type 2 diabetes and the new technology will definitely add to our understanding of the mode of action of new active molecules.

### ABBREVIATIONS

FFA	=	Free fatty acids
VLDL	=	Very low density lipoprotein
LDL	=	Low density lipoprotein
HDL	=	High density lipoprotein
CDH	=	Coronary heart disease
PPAR	=	Peroxisome proliferator activated receptor

RXR	=	Retinoid X receptor
LPL	=	Lipoprotein lipase
apoA-I	=	Apolipoprotein A-I
apoA-II	=	Apolipoprotein A-II
apoC-III	=	Apolipoprotein C-III
EC <sub>50</sub>	=	Efficient concentration at which 50% maximum effect is obtained
MED <sub>40-60</sub>	=	Minimal effective dose needed to obtain 40 – 60 % <i>in vivo</i> effect
PPRE	=	Peroxisome proliferator activated receptor response element
SPA	=	Scintillation proximity assay
LIC	=	Ligand-induced complex formation assay
CARLA	=	Co-activator-dependent receptor ligand assay
LBD	=	Ligand binding domain
DBD	=	DNA binding domain
apoE3	=	Apolipoprotein E3
PCR	=	Polymerase chain reaction
APC	=	Adenomatous polyposis coli tumour suppressor gene

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