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EFFETS HEPATOPROTECTEURS DE PPARα :

Rôle Physiopathologique et Bases Moléculaires des Activités de PPARα dans L'inflammation Aiguë et la Stéatohépatite Non Alcoolique

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Kochanym Rodzicom (To my parents)

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Résumé

La stéatohépatite non alcoolique (NASH) est une maladie du foie à évolution clinique grave, dont la prévalence est en constante progression. La stéatohépatite non alcoolique est caractérisée par un dépôt excessif de lipides dans les hépatocytes (stéatose) associé à une inflammation chronique, au contraire de la stéatose hépatique (NAFLD), manifestation initiale mais bénigne d'un dérèglement métabolique. Le NASH augmente le risque de progression vers la fibrose , la cirrhose et le carcinome hépatocellulaire et ne peut être soigné que par une greffe hépatique. Le risque de développer un diabète de type 2 est aussi significativement augmenté chez les patients atteints de NASH.

PPARα (Peroxisome Proliferator-Activated Receptor α) est un récepteur nucléaire connu pour réguler l'utilisation des acides gras dans le foie et réprimer les voies de signalisation pro-inflammatoires. L'activation pharmacologique de PPARα par des composés de type fibrate protége de la stéatohépatite induite par un régime. Néanmoins, la contribution relative des activités métabolique et anti-inflammatoires de PPARα dans la protection vis-à-vis de la NASH reste inconnue.

Nous avons conçu un mutant de PPARα dont l'activité de liaison à l'ADN est abolie. La comparaison de ses activités transcriptionnelles in vitro avec le PPARα non muté démontre que les activités de contrôle du métabolisme sont abolies pour ce mutant, alors que les activités anti-inflammatoires restent intactes. La ré-expression de PPARα sauvage ou PPARα muté dans le foie par le biais d'une hydroporation ou l'usage de particules virales de type AAV8 démontre que, dans des modèles aigus et chroniques d'inflammation, les effets anti-inflammatoires de PPARα, et non ses effets métaboliques, résultent de mécanismes indépendants de la liaison à l'ADN *in vivo*.

Dans cette étude, nous montrons donc pour la première fois que PPARα inhibe la progression de la stéatose vers le NASH et la fibrose par un mécanisme anti-inflammatoire direct, indépendant de son effet sur le métabolisme lipidique hépatique.

Les mots clés : PPARα/PPRE/NASH/fibrose

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HEPATOPROTECTIVE EFFECTS OF PPARα:

Molecular Basis and Pathophysiological Role of PPARα in Acute Inflammation and Non-Alcoholic Steatohepatitis

Summary

Non-alcoholic fatty liver disease (NAFLD) is an increasingly prevalent liver condition characterized by excessive lipid deposition in the hepatocytes (steatosis) progressing to non-alcoholic steatohepatitis (NASH) is hallmarked by chronic inflammation. NASH markedly increases the risk of progression towards liver fibrosis, cirrhosis and hepatocellular carcinoma. The nuclear peroxisome proliferator-activated receptor alpha (PPARα) regulates hepatic fatty acid utilization and represses pro-inflammatory signaling pathways. Pharmacological activation of PPARα reverses diet-induced steatohepatitis, nevertheless, the relative contribution of lipid normalizing vs. anti-inflammatory activities of PPARα in NASH progression is unknown. Liver-specific expression of wild type or DNA binding-deficient PPARα in acute and chronic models of inflammation demonstrated that PPAR's anti-inflammatory, but not metabolic activities, result from DNA binding-independent mechanisms *in vivo*. We further show that PPARα inhibits the transition from steatosis toward NASH and fibrosis through a direct, anti-inflammatory mechanism independent of its effect on hepatic lipid metabolism.

Keywords: PPARα/PPRE/FAO/NASH/fibrosis

Résumé Détaillé de la Thèse

EFFETS HEPATOPROTECTEURS DE PPARα :

Rôle Physiopathologique et Bases Moléculaires des Activités de PPARα dans L'inflammation Aiguë et la Stéatohépatite Non Alcoolique

INTRODUCTION

Anatomie et fonctions du foie

Le foie est l'organe le plus volumineux du corps humain. Il joue un rôle essentiel dans le métabolisme des acides gras, du cholestérol et des acides biliaires au cours des différentes phases nutritionnelles. Le foie a également un rôle majeur dans le maintien de l'homéostasie glucidique via la régulation de la néoglucogenèse (production de glucose à partir de substrats carbonés non glucidiques), de la glycogenèse (formation de glycogène à partir de glucose) et de la glycogénolyse (dégradation du glycogène en glucose). Par ailleurs, le foie est le site du catabolisme des acides aminés et il représente l'organe clé de synthèse de la plupart des protéines plasmatiques, telles que l'albumine, les protéines de la phase aiguë de l'inflammation ou les facteurs de coagulation. Enfin, le foie est le principal site du métabolisme des xénobiotiques (biotransformation) et il joue un rôle central dans les processus de détoxification et d'élimination des déchets.

Au niveau structural, le foie humain se compose de deux lobes principaux, le lobe gauche et le lobe droit, séparés par le ligament falciforme. Le foie est approvisionné en sang par deux sources principales : près de 75 % de l'apport sanguin au foie proviennent de la veine porte qui transporte le sang veineux, partiellement désoxygéné, de l'appareil digestif au foie. Par conséquent, le sang provenant de la veine porte est riche en nutriments tels que les acides gras, le glucose et les acides aminés, et il contient certaines hormones telles que l'insuline et le glucagon. Les 25% de l'apport sanguin restant proviennent de l'artère hépatique qui fournit essentiellement un sang riche en oxygène.

Au niveau histologique, le foie est divisé en unités fonctionnelles appelées lobules hépatiques. Initialement décrites par Kiernan en 1833, ces sous-unités présentent une structure hexagonale, à peu près cylindrique, contenant des travées d'hépatocytes qui sont orientés de façon radiale et qui partent d'une veine centrale. A chacun des coins du lobule se trouve un espace interlobulaire ou espace porte de Kiernan qui contient 3 types de vaisseaux (ou triade porte), à savoir une branche de l'artère hépatique, une branche de la veine porte et un canal biliaire interlobulaire.

Outre cette architecture classique du foie en lobules hépatiques, Rappaport a proposé, en 1954, le concept d'unités fonctionnelles appelées acinus hépatique. L'acinus va définir trois zones concentriques dont l'oxygénation est différente. La zone 1 la plus proche de l'espace porte sera la plus vascularisée et la plus riche en échanges alors que la zone 3 proche de la veine centrale sera la plus pauvre en oxygène. Ainsi, la libération de glucose, l'oxydation des acides gras, l'utilisation des acides aminés ou la formation de la bile se produisent principalement dans la zone périportale, tandis que l'utilisation du glucose et la détoxification des xénobiotiques se font principalement dans la zone péricentrale.

Le foie est constitué de nombreux types de cellules parmi lesquels les hépatocytes sont les plus nombreux, représentant plus de 60 % de la population totale. Les 40% restants sont représentés par les cellules de Kupffer, les cellules endothéliales sinusoïdales et les cellules stellaires. Les cellules de Kupffer représentent environ 15% des cellules du foie, ce sont des macrophages dérivés des monocytes circulants, attachés aux cellules endothéliales. Les cellules endothéliales sinusoïdales (20 % des cellules du foie) constituent la première barrière entre le sang et les hépatocytes, et ont pour rôle de filtrer les fluides et les particules provenant de l'espace périsinusoïdal (appelé également espace de Disse). Les cellules stellaires (5% des cellules du foie), appelées également cellules de Ito, sont spécialisées dans la production de la matrice extracellulaire et jouent également un rôle important dans divers processus tels que la régénération du foie, la fibrogénèse hépatique, la régulation du flux sanguin et le stockage de la vitamine A.

Le foie et la superfamille des récepteurs nucléaires

Récepteurs nucléaires et foie

Les récepteurs nucléaires sont des facteurs de transcription, activés par la fixation de ligands, qui intègrent et traduisent des signaux physiologiques grâce à la régulation de gènes impliqués dans divers processus biologiques (contrôle du cycle cellulaire, prolifération

cellulaire, métabolisme, inflammation, détoxification…). Au niveau du foie, les récepteurs nucléaires assurent l'adaptation de la fonction hépatique à différents composants de l'alimentation en contrôlant diverses voies métaboliques et en régulant les réponses associées à l'exposition du foie à un médicament, à un stress ou au cours de la régénération hépatique. Les récepteurs nucléaires présentent un profil d'expression spécifique à certains tissus et à certaines cellules. Au niveau hépatique, la plupart des récepteurs nucléaires sont exprimés par les hépatocytes. C'est le cas des récepteurs nucléaires activés par les toxines, tels que CAR (Constitutive Androstane Receptor) ou PXR (Pregnane X Receptor), du récepteur nucléaire PPARα (Peroxisome Proliferator-Activated Receptorα) activé par les lipides, du récepteur nucléaire FXR (Farnesoid X Receptor) activé par les acides biliaires ou du récepteur nucléaire LXR (Liver X Receptor) activé par les oxystérols. Cependant, certains récepteurs nucléaires, tels que les récepteurs orphelins NUR77 et COUP-TF2 (COUP transcription factor 2), semblent être exprimés majoritairement par les cellules non parenchymateuses, à savoir les cellules de Kupffer et les cellules endothéliales.

Implication du récepteur nucléaire PPARα dans le métabolisme lipidique et l'inflammation hépatique

PPARα (Peroxisome Proliferator-Activated Receptorα), aussi appelé NR1C1, est un récepteur nucléaire, activé par un ligand, connu pour réguler l'utilisation des lipides dans les tissus présentant un catabolisme des acides gras élevé tels que le foie, le cœur et le muscle squelettique. PPARα adopte la structure tertiaire classique des récepteurs nucléaires, avec un domaine de liaison du ligand en C-terminal (LBD) et un domaine central de liaison à l'ADN (DBD), hautement conservé, permettant l'interaction de PPARα avec des éléments de réponse spécifiques sur l'ADN appelés PPRE (Peroxisome Proliferator Response Element) localisés parfois dans la région promotrice de ses gènes cibles, mais préférentiellement dans les régions distantes dites "enhancers" ou introniques.

Dans le foie, PPARα permet le maintien de l'homéostasie lipidique et glucidique, et favorise la synthèse des corps cétoniques au cours du jeûne. Ces effets transmis par PPARα s'effectuent grâce à la régulation transcriptionnelle de nombreux gènes impliqués dans l'oxydation des acides gras et dans l'utilisation énergétique. Ce processus, appelé transactivation, s'effectue via la formation d'hétérodimères entre PPARα et le récepteur nucléaire RXR (Retinoid X Receptor), permettant ainsi de réguler la transcription de gènes cibles de PPARα via la fixation à l'ADN de ces hétérodimères sur les éléments de réponse PPRE. PPARα exerce également des effets anti-inflammatoires par un mécanisme

indépendant de la fixation à l'ADN, appelé mécanisme de transrépression. En effet, PPARα peut inhiber l'expression de cytokines pro-inflammatoires en interférant négativement avec les voies de signalisation AP- 1, NFκB et C/EBPß. L'importance physiologique de PPARα dans ce mécanisme de transrépression a été démontrée dans des modèles d'inflammation induite par des cytokines, par des bactéries, ou dans des modèles d'endotoxémie induite par le LPS.

PPARα est un récepteur nucléaire activé par des ligands endogènes, tels que des phospholipides ou des acides gras polyinsaturés, ou par des ligands synthétiques représentés par la classe thérapeutique des fibrates. Chez l'homme, les fibrates sont utilisés en clinique pour diminuer le taux de triglycérides plasmatiques et augmenter les niveaux plasmatiques de HDL-C chez les patients atteints de dyslipidémie. De façon intéressante, des études récentes ont montré que les fibrates peuvent agir sur les différents stades de stéatopathies hépatiques non alcooliques (NAFLD).

Origine et développement des stéatopathies hépatiques non alcooliques (NAFLD)

Les NAFLD sont caractérisées par une accumulation de lipides (stéatose), supérieure à 5 % du poids du foie, et elles touchent 20 à 30 % de la population générale. Cependant, chez les personnes obèses, la prévalence des NAFLD peut atteindre 75 à 100 %. Les NAFLD étant la manifestation hépatique du syndrome métabolique, elles sont souvent associées à une surcharge pondérale, une dyslipidémie (concentration élevée de TG plasmatiques et diminution des taux plasmatiques de HDL-C) et une résistance à l'insuline voire un diabète de type 2. Dans la plupart des cas, les patients ont une maladie bénigne non évolutive avec présence d'une simple stéatose hépatique, souvent asymptomatique. Néanmoins, certains patients peuvent évoluer vers une stéatohépatite non alcoolique (NASH), qui se manifeste par une stéatose hépatique accompagnée par des lésions histologiques inflammatoires chroniques. Histologiquement, les lésions du NASH sont pratiquement identiques à celles de la stéatohépatite alcoolique (ASH), mais elles apparaissent en dehors de toute consommation excessive d'alcool. Traditionnellement, il a été démontré que l'accumulation de lipides représente la première étape du NASH, déclenchant par la suite un stress oxydatif, une lipotoxicité et une activation de réponses inflammatoires au niveau hépatique. Chez les individus prédisposés, le NASH peut alors ensuite évolué vers des formes plus graves d'atteinte hépatique telles que la fibrose, la cirrhose voire le carcinome hépatocellulaire.

L'inflammation est un mécanisme essentiel dans la progression des NAFLD. Ainsi, les patients développant un NASH présentent des concentrations plasmatiques plus élevées de cytokines pro-inflammatoires, telles que TNFα (Tumor Necrosis Factor alpha) et IL-6 (Interleukin-6), et de VEGF (Vascular Endothelial Growth Factor) par comparaison avec des patients sains. Au niveau du foie, les cellules immunitaires innées, telles que les monocytes et les cellules de Kuppfer, et secondairement les hépatocytes jouent un rôle essentiel dans le développement du NASH en régulant les réponses immunitaires locales par la sécrétion de cytokines et de chimiokines. Ainsi, des études récentes ont montré que la production du TNFα par les cellules de Kuppfer est cruciale pour l'induction de la phase précoce du NASH. D'autres études ont montré l'importance du TGFβ (Transforming Growth Factor beta), sécrété par les cellules de Kuppfer et les hépatocytes, dans la progression de la fibrose hépatique puisque le TGFβ active les cellules stellaires à l'origine de la synthèse de collagène.

De plus, des études récentes ont suggéré un rôle majeur des inflammasomes dans la progression des NAFLD. Les inflammasomes sont des complexes protéiques responsables de l'activation des cellules immunitaires et de la production de cytokines pro-inflammatoires en réponse à la lésion ou à la destruction d'un tissu. Parmi eux, NLRP3 est un inflammasome connu pour être activé par la production accrue de dérivés réactifs de l'oxygène (reactive oxygen species, ROS), suite à une augmentation de la peroxydation lipidique. L'activation de NLRP3 conduit à un clivage, dépendent de la caspase-1 (Casp-1), de cytokines pro-inflammatoires telles que la pro-IL-1β et la pro-IL-18 dont le rôle dans la progression de la stéatose vers le NASH et la fibrose a clairement été démontré. En effet, des études ont montré que des souris déficientes pour Casp1 ou pour IL-1β sont protégées contre le NASH induit par un régime et contre la fibrose hépatique.

OBJECTIFS DE LA THÈSE

Plusieurs études ont montré un rôle bénéfique de PPARα dans la progression des NAFLD grâce à son activité transactivatrice sur la régulation du métabolisme lipidique intrahépatique. De plus, il est connu que PPARα, via son activité transrépressive anti-inflammatoire, peut interférer avec les voies de signalisation AP1 et NFκB, voies dont l'activation dans le développement du NASH et de la fibrose semble être maintenant bien établie. Cependant, dans les effets de PPARα, la part relative de son activité anti-inflammatoire par rapport à son activité sur la régulation du métabolisme lipidique est difficile à distinguer et,

particulièrement, l'implication relative de ces deux mécanismes dans un contexte de NAFLD n'a jamais été étudiée.

Pour cela, nous avons conçu un mutant de PPARα, appelé PPARα$_{DISS,}$ dont l'activité de liaison à l'ADN est abolie, créant ainsi potentiellement un mutant incapable de posséder des propriétés transactivatrices sur la régulation des gènes du métabolisme lipidique, tout en conservant des propriétés transrépressives sur l'inflammation. Dans un premier temps, les effets de ce mutant PPARα$_{DISS}$ ont été testés et validés *in vitro* par comparaison au PPARα non muté. Dans un deuxième temps, les effets du PPARα$_{DISS}$ ont été testés *in vivo* dans des modèles d'inflammation aiguë et d'endotoxémie induite par le LPS. Enfin, dans un troisième temps, ce mutant PPARα$_{DISS}$ a été étudié chez la souris dans un contexte de NAFLD induite par un régime déficient en choline et méthionine (MCDD).

RÉSULTATS ET DISCUSSION

Pour dissocier les propriétés transactivatrices et transrépressives de PPARα, nous avons créé un mutant de PPARα, le mutant PPARα$_{DISS}$ R150Q, après substitution d'une arginine en glutamine en position 150 dans le 2ème doigt de zinc ZF2 du domaine de liaison à l'ADN (DBD). Ce mutant serait potentiellement incapable de se fixer aux éléments de réponse PPRE sur l'ADN, supprimant ainsi toute activité transactivatrice de PPARα sur ses gènes cibles.

In vitro, nous avons pu confirmer que le PPARα$_{DISS}$ ne pouvait plus se fixer aux séquences consensus DR-1 et aux éléments de réponse PPRE de PPARα sur l'ADN. En effet, le PPARα$_{DISS}$ était incapable de déclencher l'activation transcriptionnelle des gènes cibles de PPARα, tels que *Acox1* (Acyl-coA oxydase), *Cpt-1* (Carnitine palmitoyltransferase-1) et HMG-CoA Synthase (hydroxymethyl-glutaryl-CoA synthase), bloquant ainsi toute activité transactivatrice. Par contre, comme le PPARα non muté, le PPARα$_{DISS}$ pouvait conserver ses interactions avec son partenaire d'hétérodimérisation RXRα ainsi qu'avec ses protéines co-régulatrices. De plus, nous avons montré que le PPARα$_{DISS}$ pouvait toujours interagir, à la fois au niveau physique et fonctionnel, avec les protéines p65 et c-Jun du complexe NFκB ainsi qu'avec les facteurs de transcription AP-1, conservant ainsi son activité transrépressive par inhibition des voies de signalisation pro-inflammatoires. Nos résultats *in vitro* ont ainsi pu montrer que, par simple mutation créant une altération dans la structure du DBD, nous avons créé, pour la première fois, un mutant de PPARα incapable de transactivation mais

conservant ses propriétés de transrépression. Ce type de construction avait déjà été réalisé précédemment avec le récepteur aux glucocorticoïdes GR dans lequel la mutation A458T dans le domaine en doigt de zinc ZF2 avait induit un défaut de dimérisation du récepteur. Comme pour PPAR$\alpha_{DISS,}$ le mutant A458T est incapable de se lier à l'ADN tout en maintenant une activité anti-inflammatoire puissante.

Nous avons ensuite testé l'effet de ce mutant PPARα_{DISS} *in vivo* dans un modèle d'endotoxémie induite par le LPS. En effet, le LPS est un ligand de TLR4 connu pour déclencher une réponse inflammatoire aiguë rapide avec libération de cytokines pro-inflammatoires, telles que TNFα, IL1b, et IL6. Pour cela, des souris déficientes pour PPARα ont été hydroporées avec un vecteur d'expression pour le PPARα non muté ou pour le PPARα_{DISS}. L'hydroporation permet l'injection, par voie intraveineuse et en quelques secondes, d'un plasmide contenant un vecteur d'expression dans un volume de tampon égal au volume de sang total, ce qui permet de cibler spécifiquement le foie. Les souris ont ensuite été gavées par fénofibrate et injectées avec le LPS. De manière intéressante, l'activation par le fénofibrate du PPARα non muté a induit l'expression du gène de l'*Acox1*, gène cible de PPARα, alors que le PPARα_{DISS} était incapable de l'induire. Par contre, la restauration du PPARα non muté ou celle du PPARα_{DISS} a permis de bloquer l'inflammation induite par le LPS en réprimant l'expression de gènes clés de la phase aiguë de l'inflammation tels que *Saa* (Serum Amyloid A) et *fibrinogen-alpha*, ainsi que l'expression de cytokines pro-inflammatoires tels que *Tnfα*, *Il1b* et *Il6*. Ainsi, nous avons pu valider *in vivo* la fonctionnalité du mutant PPARα_{DISS} sur la transrépression dans un contexte d'inflammation aiguë induite par le LPS et, plus largement, nous avons confirmé la possibilité d'étudier distinctement les effets de PPARα sur la transrépression indépendamment de la transactivation.

Ces effets dissociés de PPARα ont ensuite été étudiés dans un contexte physiopathologique chronique de NAFLD. En effet, les NAFLD englobent des atteintes hépatiques diverses, allant de la simple stéatose hépatique au NASH (associant stéatose et inflammation chronique), pouvant évoluer ensuite vers la fibrose. Plusieurs études ont montré que l'activation pharmacologique de PPARα protège les souris sauvages, et non les souris déficientes en PPARα, de la progression de la stéatose vers le NASH puis la fibrose au cours d'un régime MCD (déficient en méthionine et en choline), régime connu pour mimer la progression des stéatopathies chez l'homme. Ainsi, grâce au mutant PPARα_{DISS}, nous souhaitions évaluer, indépendamment de la transactivation de gènes clés du métabolisme lipidique hépatique, la part relative de l'activité transrépressive anti-inflammatoire de PPARα dans son rôle protecteur lors de la progression des NAFLD. Pour cela, des souris déficientes

pour PPARα ont été injectées avec des particules virales AAV8 (Adeno-associated virus 8) exprimant le PPARα non muté ou le PPARα$_{DISS}$, permettant ainsi d'exprimer de manière stable dans le temps et de manière spécifique dans le foie le PPARα non muté ou le PPARα$_{DISS}$. Ces souris ont ensuite été mise au régime MCDD durant 3 semaines et traitées pendant les 5 derniers jours par le fénofibrate. Contrairement au PPARα non muté, l'expression du PPARα$_{DISS}$ dans le foie n'a pas montré d'effets bénéfiques sur la stéatose hépatique, ce qui était corrélé avec l'absence de régulation des gènes du métabolisme lipidique intrahépatique tels que *Acox1*, *Bien* et *Cyp4a10*, suite à l'activation par le fénofibrate. Par contre, comme le PPARα non muté, l'activation du PPARα$_{DISS}$ a induit une diminution d'expression des cytokines pro-inflammatoires telles que *proIl1b*, l'*Il6* et le *Tnfα,* connues pour être essentielles dans la progression de la stéatose vers le NASH. De manière intéressante, après activation par le fénofibrate, nous avons également montré que l'expression du PPARα$_{DISS}$ a induit une diminution de l'expression de gènes impliqués dans le processus de fibrose tels que *Col1α1* (collagen1 α1) et *Timp1* (Tissue inhibitor of metalloproteinase 1), de manière comparable au PPARα non muté, et que cet effet était associé à une diminution quantitative du collagène hépatique. Ainsi, la protection de PPARα contre la progression du NASH vers la fibrose serait uniquement liée à un mécanisme transrépresseur anti-inflammatoire, indépendant de tout effet sur le métabolisme lipidique intrahépatique. Dans une précédente étude, il avait été suggéré que PPARα pouvait contrecarrer le développement de la fibrose en diminuant les stimuli profibrotiques issus de la peroxidation lipidique. Dans notre étude, nous montrons que seul le mécanisme de transrépression, soit en induisant directement la répression de gènes profibrotiques et/ou en diminuant en amont les voies de signalisation pro-inflammatoires, suffisait pour bloquer la progression vers la fibrose, de façon indépendante de toute modulation du métabolisme lipidique. Cette conclusion est en accord avec les études récentes démontrant un rôle majeur de l'inflammation et des inflammasomes tels que NRLP3 dans la progression du NASH vers la fibrose.

CONCLUSION

En conclusion, nous avons pu montrer, grâce à l'utilisation du mutant PPARα$_{DISS,}$ que le mécanisme de transrépression réalisé par PPARα est essentiel pour bloquer les mécanismes inflammatoires aigus, par exemple au cours de l'endotoxémie induite par le LPS, mais également pour contrecarrer les réponses inflammatoires chroniques impliquées

dans l'évolution des NAFLD, particulièrement lors la transition vers le NASH et la fibrose. Cette étude laisse entrevoir l'importance de la synthèse de nouveaux ligands de PPARα plus axée sur leur activité transrépressive dans l'objectif, par exemple, de leur utilisation dans des stades plus tardifs de NAFLD.

Contents

Chapter 1

GENERAL INTRODUCTION

LIVER FUNCTION AND ANATOMY

The liver is the largest organ of the human body playing a central role in the metabolism of fatty acids, cholesterol and bile acids under different nutritional states. The liver maintains carbohydrate homeostasis via gluconeogenesis (generation of glucose from non-carbohydrate carbon substrates), glycogenesis (formation of glycogen from glucose), glycogenolysis (breakdown of glycogen to glucose) and glycolysis (conversion of glucose into pyruvate). The liver is the site of amino acid catabolism and serum protein synthesis, including albumin, glycoprotein transport protein, acute phase proteins and coagulation factors. Additionally, liver is the major site of biotransformation and defence against metabolic wastes and xenobiotics.

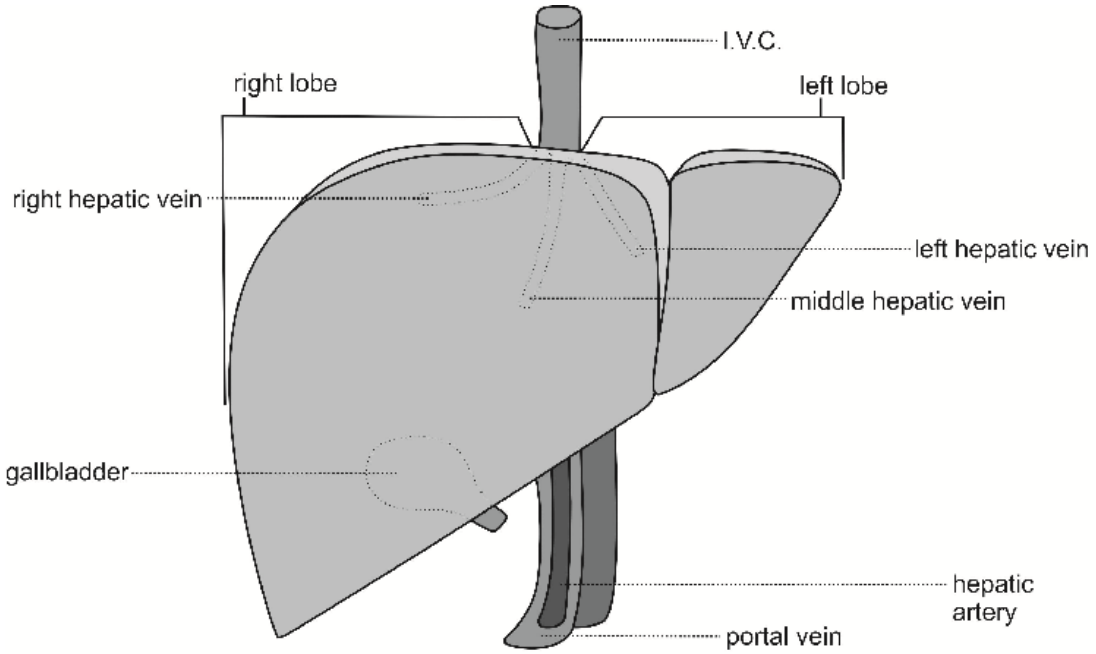

Figure 1. Liver anatomy. Diagrammatic presentation of segmental liver anatomy emphasizing intrahepatic ducts and blood vessels; inferior vena cava (I.V.C).

Macroscopically, the middle hepatic vein divides the liver into functional left and right lobes, whereas upper and lower segments of the liver are separated by the portal vein and its branches (Figure 1). However, anatomical differences exist between species [1]. The liver has two distinct blood supplies. Almost 75% of the blood supplied to the liver is delivered by the portal vein which conducts partially deoxygenated venous blood from the gastrointestinal tract and spleen [2]. Hence, portal vein blood is rich in nutrients such as fatty acids, glucose

and amino acids as well as certain hormones including insulin and glucagon. The hepatic artery delivers highly oxygenated blood to the corresponding lobes of the liver that accounts 20-25% of total afferent blood volume [3].

FUNCTIONAL ZONATION OF LIVER CELLS

Histologically, the liver is divided into functional units, called hepatic lobules. The classic hexagonal lobule was for the first time described by Kiernan in 1833 [4]. The architecture of the classic lobule is typically hexagonal in cross section, with a central vein (a branch of the hepatic vein) at its centre and portal areas at its peripheral corners forming portal triads together with the bile duct and hepatic artery (Figure 2) [3]. The concept of diamond shape, functional unit called liver acinus was proposed by Rappaport in 1954 [5]. Accordingly, the oxygen-rich blood is supplied by hepatic arteries (zone 1) and is drained at two peripheral central veins through the sinusoidal blood flow thus hepatocytes are poorest oxygenized near to the central vein (zone 3).

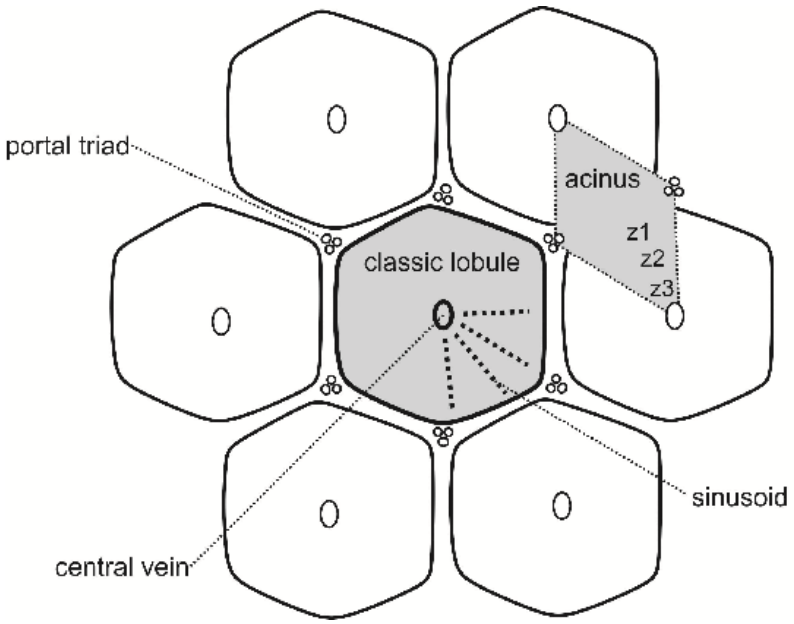

Figure 2. Liver microanatomy and zonation. Microscopically, liver parenchyma is divided in functional units, classified according to the localization of the central vein and portal triad defined by the portal vein, bile duct, and hepatic artery (classic lobule) or according to the oxygen supply (acinus); zone 1, 2, 3 (z1, z2, z3, respectively).

Functional heterogeneity of hepatic parenchyma is linked to the position of the cell within the functional unit of the tissue and blood supply [6]. Those zonal differences are caused by gradients in oxygen, substrate and hormone availability and mediator levels that imprint in the transcriptional regulation of gene sets involved in various metabolic processes [7]. According to the model of metabolic zonation, glucose release, fatty acid oxidation, amino acid utilization and bile formation occur mainly in the periportal zone, whereas glucose utilization and xenobiotic detoxification take place in the pericentral zone (Table 1) [8].

Periportal Zone	Pericentral Zone
Glucose release	Glucose uptake
Oxidative energy metabolism FAO, TCA, respiratory chain	Glycogen synthesis Glycolysis
Amino acid utilization amino acid conversion to glucose amino acid degradation	Lipogenesis
Ureagenesis from amino acid nitrogen protein synthesis (blood clotting factors, plasma proteins, APR proteins), ammonia detoxification	Ureagenesis from ammonia nitrogen
Oxidative protection	Biotransformation of drugs
Cholic acid excretion	
Bilirubin excretion	Bilirubin excretion

Table 1. The zonation of hepatocyte functions. Hepatocytes concentrically arranged around the portal veins (Periportal Zone) and central veins (Pericentral Zone); fatty acid oxidation (FAO); tricarboxylic acid cycle (TCA); acute phase response (APR). Adopted from Demetris AJ, Functional anatomy of the normal liver, 2011.

The liver consists of at least 15 different cell types amongst which hepatocytes are the most numerous and comprise 60% of the total cell population. Remaining non-parenchymal cells of the liver such as Kupffer cells (KCs), sinusoidal endothelial cells (SECs), and hepatic stellate cells (HSCs) represent 3-20% of the total population each [5]. KCs comprising about 15% of the liver cells are specialized macrophages adherent to endothelial cells of the sinusoids [9]. SECs (20% of liver cells) constitute the primary barrier between blood and hepatocytes by filtering fluids and particles coming through the perisinusoidal space (known also as Disse space) [10]. HSCs (5% of total liver cells), also called Ito cells, are specialized in the production of extracellular matrix and play a role in liver regeneration and hepatic fibrogenesis as well as control the microvascular tone and vitamin A metabolism [11].

THE LIVER AND THE NUCLEAR RECEPTOR SUPERFAMILY

Nuclear receptors in the liver

Nuclear receptors (NRs) are ligand-activated transcription factors that integrate and translate physiological signals into regulation of genes involved in biological processes such as cell cycle control, cell proliferation, metabolism, inflammation and detoxification. NRs reveal tissue-specific and cell-specific distribution patterns. The majority of identified NRs has been found to be expressed either in liver parenchymal or non-parenchymal cells. Among them

toxin-activated NRs, such as constitutive androstane receptor (CAR) and pregnane X receptor (PXR) and lipid and bile acid-activated NRs, including proliferator-activated receptors (PPAR), liver X receptors (LXR) and the farnesoid X receptor (FXR) are ubiquitously expressed in parenchymal liver cells, underlying their importance in xenobiotic clearance by the liver, lipid and bile acid metabolism, and inflammation [12-14]. Recent studies show that in mice liver-expressed NRs are more abundantly expressed in hepatocytes than in non-parenchymal cells, such as SECs and KCs with the exception of the orphan receptors NUR77 and COUP-TF2 which appear more strongly expressed in non-parenchymal cells [15].

NRs ensure the adaptation of liver function to distinct components of the diet by controlling a large variety of metabolic pathways and integrating responses to drug exposure, liver injury and regeneration. A better understanding of NR mechanisms of action may provide new perspectives for the treatment of a wide range of liver pathologies. In-depth structure-to-function analysis of the NR superfamily is discussed in **chapter 2** entitled **'General molecular biology and architecture of nuclear receptors'**. NR modes of action are reviewed with a special attention to their interaction with ligands, DNA and the regulation of NR activity by a range of co-regulators. Moreover, in this chapter different models of NR-dependent transcriptional repression and activation of genes are discussed.

The role of PPARα in hepatic lipid turnover and inflammation

Amongst the NRs expressed in liver parenchymal cells, PPARα plays a central role in lipid homeostasis via controlling hepatic fatty acid turnover and lipoprotein metabolism [16]. Moreover, pharmacologically activated PPARα may counteract the acute phase response by mechanisms operating in the liver and hamper chronic inflammation in murine models of nutritional steatohepatitis [17, 18].

The work presented in this thesis focuses particularly on the role of PPARα in lipid metabolism and inflammation in the liver. PPARα's molecular mechanisms of action in the liver and its impact on de novo fatty acid synthesis (lipogenesis), FA oxidation (FAO) and lipoprotein metabolism are reviewed in **chapter 3 'Molecular mechanism of PPARα action and its impact on lipid metabolism and inflammation'**. Special attention is given to our understanding of PPARα-driven gene activation (transactivation) in the regulation of gene clusters related to FAO and PPARα-dependent transcriptional repression (transrepression) of pro-inflammatory genes. Finally, current knowledge on the pathophysiological role of PPARα in non-alcoholic fatty liver disease (NAFLD) is discussed based on existing pre-clinical and clinical data.

Origin and development of NAFLD

NAFLD is hallmarked by lipid accumulation exceeding 5% of liver weight and affects 20-30% of the general population [19]. NAFLD is the hepatic manifestation of the metabolic syndrome thus is strongly associated with central obesity, dyslipidemia (elevated plasma TG, reduced HDL cholesterol) and insulin resistance [20]. Accordingly, in obese individuals the prevalence of NAFLD ranges from 75-100%. In most patients NAFLD remains asymptomatic, nevertheless some individuals may progress to non-alcoholic steatohepatitis (NASH), manifested by steatosis accompanied by chronic inflammation [21]. Histologically NASH is

virtually indistinguishable from alcoholic steatohepatitis (ASH), but it appears in the absence of significant alcohol consumption [22].

According to the multiple-hit model of NAFLD/NASH pathogenesis, lipid accumulation represents the first hit in NASH initiation, triggering oxidative stress, lipotoxicity and subsequent activation of hepatic inflammatory responses that further progress, in predisposed individuals, to more severe forms of liver pathology such as fibrosis, cirrhosis and hepatocarcinomas (Figure 3) [22]. Recent studies suggested an important role of inflammasomes, which serve as pattern recognition receptors responsible for activation of immune cells in response to signals released upon tissue injury and death [23]. Among them, NLRP3 is known to be activated by increased generation of ROS derived from enhanced lipid peroxidation. NLRP3 activation leads to caspase-1 (*Casp-1*)-dependent cleavage of effector pro-inflammatory cytokines, such as pro-IL-1β and pro-IL-18 [19]. IL-1β has, in turn, a prominent role in the progression from steatosis to steatohepatitis and liver fibrosis [24].

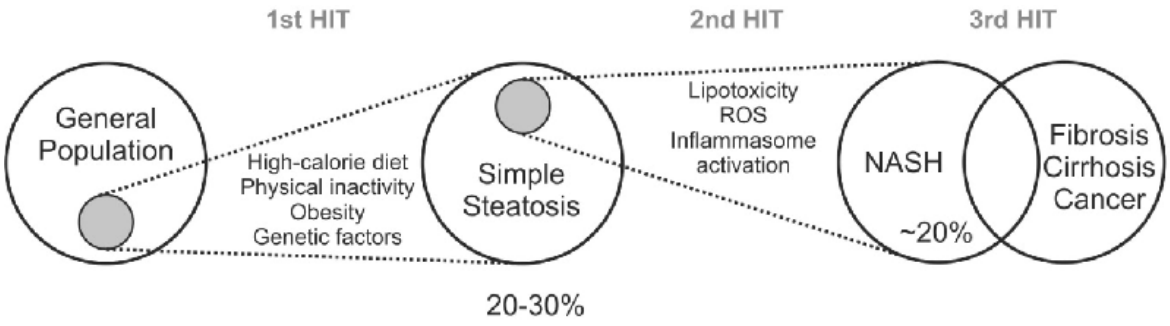

Figure 3. Progression of NAFLD. Development of liver steatosis is often associated with several risk factors including high-calorie diet, physical inactivity and genetic factors that may result in insulin resistance and excessive lipid accumulation in the liver that may affect 20-30% of general population (1st hit). Intrahepatic lipids yield lipotoxic compounds and ROS that further activate a pro-inflammatory response. At least 20% of affected individuals will develop NASH (2nd hit) which may progress to fibrosis, cirrhosis and liver cancer (3rd and further hits).

In line with that, studies carried out in *Casp1*$^{-/-}$ and *Il-1β*$^{-/-}$ mice showed protection from dietary-induced steatohepatitis and liver fibrosis [24, 25]. Moreover, serum levels of cytokines, such as tumor necrosis factor (TNF), interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) are significantly elevated in patients with NASH compared to the healthy controls [26]. Innate immune cells, such as monocytes and KCs, are likely to play an essential role in NASH development by orchestrating local immune responses through the secretion of cytokines and chemokines. Accordingly, recent studies show that KC-specific production of TNF is crucial for the induction of early phase NASH [27]. Moreover, under pro-inflammatory stimuli either KCs or hepatocytes may synthesize and release transforming growth factor beta (TGFβ), which in turn activates hepatic stellate cells (HSCs) to produce collagen [28, 29]. Collagen deposition in between hepatocytes leads to hepatic fibrosis that may progress to more severe forms of chronic liver disease, such as cirrhosis, hepatocellular carcinoma and finally liver failure [30].

APPROACHES USED IN THE THESIS

Chapter 4 contains a brief description of selected experimental procedures and original techniques developed and optimized to thoroughly validate the hypothesis formed in this thesis.

OBJECTIVES OF THE THESIS

Peroxisome proliferator-activated receptor alpha (PPARα/NR1C1) is a ligand-activated nuclear receptor controlling lipid utilization in tissues such as liver, heart and skeletal muscles. Through the transcriptional regulation of gene clusters controlling fatty acid oxidation (FAO) and energy utilization in the liver, PPARα maintains lipid and glucose homeostasis, and promotes ketone body synthesis upon food deprivation [16].

PPARα adopts the classical tertiary structure of nuclear receptors, with a C-terminal ligand binding domain (LBD) and a central, highly conserved DNA-binding domain (DBD) allowing the interaction of PPARα with specific DNA sequence elements termed Peroxisome Proliferator Response Elements (PPRE). PPARα forms chromatin-bound, transcriptionally activatable heterodimers with Retinoic X Receptors (RXRs) which are thought to play a major role in PPARα-mediated transactivation of its target genes [33] (see **chapter 3**). PPARα also exerts anti-inflammatory effects during hepatic and systemic inflammation by repressing pro-inflammatory cytokine and acute phase gene expression through a tethering-based crosstalk with the AP-1, NFκB and C/EBPβ signaling pathways [18, 31, 32] (this is also broadly discussed in **chapter 3**). The physiological importance of PPARα-driven transrepression has been demonstrated in cytokine-induced inflammation and the LPS model of endotoxemia [18].

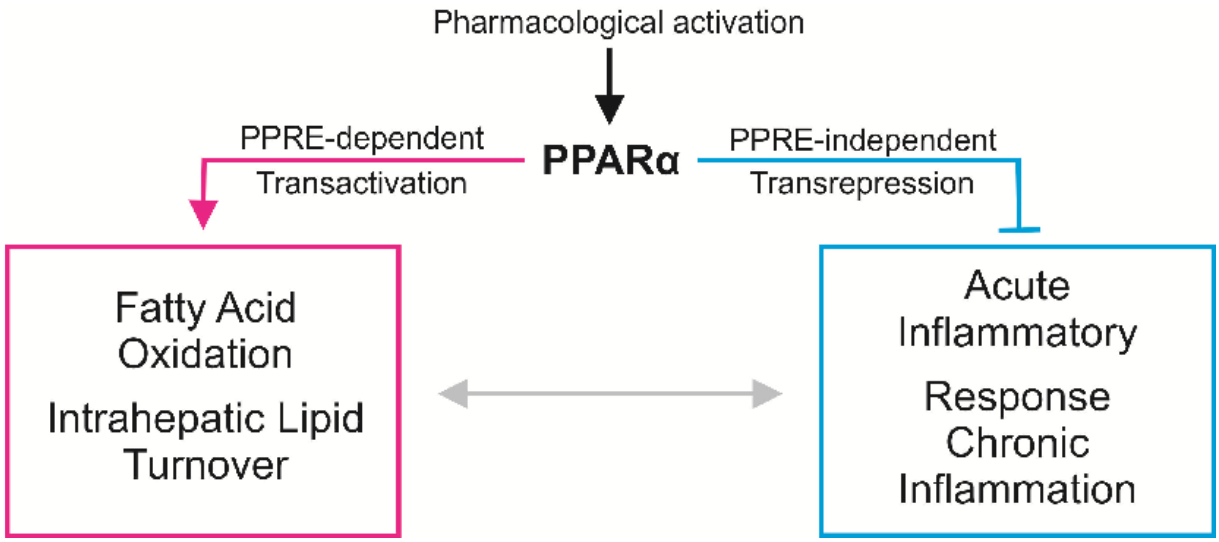

Figure 4. The concept of dissociated PPARα activities. The goal of this study is to investigate the role of dissociated PPARα activities to establish the role of PPARα-driven transrepression vs. transactivation in the transcriptional control of genes related to FAO and inflammation under pro-inflammatory stimuli as well as a possible cross-talk that may occur between 'metabolic' and anti-inflammatory PPARα modes.

Prior studies documented a strong correlation between PPARα activity, intrahepatic lipid accumulation and development of more severe liver manifestations, such as steatohepatitis and fibrosis [17, 34]. Moreover, as previously shown, PPARα interferes with AP1 and NFκB signaling pathways, which appear to be activated during the development of dietary steatohepatitis and liver fibrosis [35, 36]. Nevertheless, the relative contribution of lipid normalizing vs. anti-inflammatory activities of PPARα in hepatic physiopathology has not yet been studied.

The hypothesis of this work was that the metabolic actions of PPARα essentially result from PPRE-dependent gene activation (transactivation), whereas PPARα's ability to counteract inflammatory response stems from its transrepressing properties, in DNA binding-independent manner. Based on early *in vitro* characterization performed in our laboratory **(R. Mansouri, Thesis, 2007)**, we generated a PPARα mutant unable to bind PPRE motifs, but harbouring wild type-like interactions with co-regulator proteins and pro-inflammatory transcription factors. PPRE-independent activities of PPARα were broadly investigated *in vitro* and *in vivo* in murine models of acute inflammation/acute phase response and in the model of dietary-induced steatohepatitis. This work is introduced in **chapter 5 'PPARα inhibits progression of steatohepatitis to fibrosis via a DNA binding-independent mechanism'**.

CONTROL OF HEPATIC TRIGLYCERIDE METABOLISM BY LRH-1

As an important contribution to this thesis, the function of nuclear receptor liver receptor homolog-1 (LRH-1/NR5A2) in NAFLD and NASH development is discussed in **chapter 6** entitled **'LRH-1 plays a central role in hepatic triglyceride metabolism'**. This work, performed in collaboration with **Departments of Pediatrics and Medicine of University Medical Center Groningen, The Netherlands**, focuses on the role of LRH-1 in regulation of intrahepatic lipid turnover and unravels a functional cross-talk between LRH-1- and PPARα signaling in terms of fatty acid β-oxidation and ketogenesis. The determination of LRH-1 molecular mechanisms of action in regulation of PPARα's transcriptional activity was an essential contribution of the study performed within this thesis.

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Chapter 2

GENERAL MOLECULAR BIOLOGY AND ARCHITECTURE OF NUCLEAR RECEPTORS

ABSTRACT

Nuclear receptors (NRs) regulate and coordinate multiple processes by integrating internal and external signals, thereby maintaining homeostasis in front of nutritional, behavioral and environment challenges. NRs exhibit strong similarities in their structure and mode of action: by selective transcriptional activation or repression of cognate target genes, which can either be controlled through a direct, DNA binding-dependent mechanism or through crosstalk with other transcriptional regulators, NRs modulate the expression of gene clusters thus achieving coordinated tissue responses. Additionally, non genomic effects of NR ligands appear mediated by ill-defined mechanisms at the plasma membrane. These effects mediate potential therapeutic effects as small lipophilic molecule targets, and many efforts have been put in elucidating their precise mechanism of action and pathophysiological roles. Currently, numerous nuclear receptor ligand analogs are used in therapy or are tested in clinical trials against various diseases such as hypertriglyceridemia, atherosclerosis, diabetes, allergies and cancer and others.

Keywords: transcriptional regulation/nuclear receptors/coactivators/corepressors/structure

INTRODUCTION

The nuclear receptor superfamily comprises evolutionarily related transcription factors fulfilling multiple regulatory functions in growth, development and homeostasis. Nuclear receptors share a common architecture and functional behavior. The effector function of nuclear receptors is to modulate transcription through several distinct mechanisms, which include both transactivation and transrepression activities upon receptor-specific ligand binding. Nuclear receptors can also be the targets of other signaling pathways that modify the receptor, or their transcriptional comodulators, post-translationally and affect their activity and functions. According to phylogenetic studies, nuclear receptors emerged long before the divergence of vertebrates and invertebrates, during the earliest metazoan evolution [1]. The first cloned human receptors were the glucocorticoid receptor (GR/NR3C1, [2,3]) together with the estrogen receptor (ER) [4,5] and the thyroid hormone receptor (T3R/NR1A1, [6,7]). Forty eight nuclear receptors have since been identified in the human [8].

Nuclear receptors share a common structural organization which defines this gene superfamily (Figure 1). The N-terminal domain is highly variable depending on the receptor and contains a ligand-independent transactivation domain termed Activation Function 1 (AF-1). The most conserved central region is the DNA-binding domain (DBD), which contains the P-box, a short motif responsible for direct DNA interaction and DNA-binding specificity. Additional sequences in the DBD are involved in the homo- or heterodimerization of nuclear receptors. Nuclear receptors bind to sequence-specific elements located not only in the vicinity of target gene promoters, but also in intronic and enhancer regions, either as monomers (Nor1/NR4A3), as homodimers such as the steroid receptors [GR/NR3C1, estrogen receptors (ERα/NR3A1 and ERβ/NR3A2), progesterone receptor (PR/NR3C3), mineralocorticoid receptor (MR/NR3C2), androgen receptor (AR/NR3C4)] and retinoid X receptors (RXRα/NR2B1, RXRβ/NR2B2, RXRγ/NR2B3), or as heterodimers with RXRs. The DBD and the C-terminal ligand-binding domain (LBD) are linked by the hinge region [9]. The C terminus of NRs harbors several functionally critical motifs, such as the activating function 2 (AF-2), conferring to many NRs a ligand-dependent transcriptional activity, a strong dimerization interface and a ligand binding pocket (LBP). The in-depth structural nuclear receptor architecture is delineated further in this review.

Nomenclatures of the nuclear receptor family have been proposed according to different criteria. Based on the sequence alignment of the two well-conserved domains (DBD and LBD) and phylogenetic tree construction, the nuclear receptor gene family has been divided into six subfamilies. Interestingly and importantly, a correlation exists between DNA-binding and dimerization abilities of each classified nuclear receptor and its phylogenetic position. Subfamily 1 comprises nuclear receptors forming heterodimers with RXR (T3Rs:NR1A; RARs: NR1B; VDR: NR1I1; PPARs: NR1C; RORs: NR1F: Rev-erbs: NR1D; CAR: NR1I3; PXR: NR1I2; LXRs: NR1H). Subfamily 2 is formed by HNF4s: NR2A1&2; COUP-TFs: NR2F; RXRs: NR1B. Subfamily 2 members can function in two configurations, either as homodimers or as heterodimers. Subfamily 3 includes the above mentioned steroid hormone receptors. Subfamily 4 contains the nerve growth factor-induced clone B group of orphan receptors NGFI-B/Nur77/NR4A1, Nurr1/NR4A2, and NOR1/NR4A3. The small subfamily 5 includes the steroidogenic factor 1 (SF1/NR5A1) and receptors related to Drosophila FTZ-F1 (LRH1/NR5A2). The sixth subfamily comprises only the GCNF1 receptor. Finally, subfamily 0 encompasses 2 atypical nuclear receptors lacking the DBD (Dax1/NR0B1 and SHP/NR0B2), thereby displaying constitutive dominant-negative activities [10].

Another functional classification according to the ligand-binding properties splits the superfamily of nuclear receptors into three groups. The most characterized subfamily called thyroid/steroid hormone receptor subfamily comprises ER, AR, PR, MR and GR and also includes the thyroid receptors T3Rs, VDR, and RARs. The second 'orphan' subfamily is composed by nuclear receptors for which regulatory molecules have not been identified so far. They are represented by NR4 receptors and COUP-TFs. The function and molecular mechanism of action for many 'orphan' receptors is only poorly investigated. The third subfamily of nuclear receptors is known as 'adopted' orphan receptors. Members of this subfamily were initially characterized as 'orphans' and afterwards, natural ligands have been identified that convey physiological functions. These nuclear receptors are sensors of the metabolic status of cells, organs and the whole body and trigger responses to xenobiotics, dietary signals, diatomic gases and metabolites. In this class are found Rev-erbα and β, PPARs, LXRs, FXRs, RORs, PXR and CAR.

The ability of nuclear receptors to be regulated by natural or synthetic molecules have led to intensive efforts to target nuclear receptors therapeutically. However, many currently available ligands have several deleterious side-effects, many of which seem to be related to their transactivating properties. It seems to be essential to determine the importance of positive and negative gene regulation in conferring the therapeutic benefits of nuclear receptor ligands in disease models. In this review we will discuss the relationship between the molecular structure and the molecular action of nuclear receptors.

STRUCTURAL FEATURES OF NUCLEAR RECEPTORS

Nuclear receptors reveal characteristic protein architecture that consists of five to six domains of homology designated A to F, starting from N-terminus to C-terminus of protein. The weakest conservancy is observed in the N-terminal A/B domain, D or hinge domain, and F region at the C-terminus which is not present in all nuclear receptors. The DBD and LBD are the most highly conserved domains (Figure 1). The most recent structural studies [11,12] of RXR heterodimers bound to DNA showed asymmetric complexes of 150-200Å, with LBDs being located on one side of the DNA, 5' of the DNA response element (Figure 2). The hinge region plays an important structural role by specifying the relative orientation of the DBD with respect to the LBD.

A/B domain

The poorly structurally defined N-terminal A/B region reveals a strong diversity among nuclear receptors and because of its high mobility, its tertiary structure has not been elucidated so far. Isoform-specific differences in amino termini are observed for several NRs and these sequence variations may induce differential binding affinities to response elements and/or with members of the transcription initiation complex, distinct transcriptional activities and different in vivo roles (see for examples [13-18]).

The A/B domain contains the activation function 1 (AF-1) which is ligand-independent. Hydrogen/deuterium exchange mass spectrometry of PPARγ revealed that the ordering of A/B portion is not substantially changed upon ligand binding [11]. By contrast, the N-terminus of T3Rβ1 may transmit thyroid hormone-dependent signaling to the general transcriptional machinery by a direct interaction of the receptor with transcription factor IIB (TFIIB, [13,19]).

Moreover, the N-terminal region is an interaction surface for multiple transcriptional coregulatory proteins: steroid receptor coactivator-1 (SRC-1/NCoA1), steroid receptor coactivator-2 (SRC-2/TIF2/NCoA2), p300 and CBP enable a functional synergism between AF-1 and AF-2 regions of steroid receptors, PPARγ or RARs and thus cooperatively enhances transactivation [20-23]. In addition, co-regulator-linked interactions with the N-terminal and C-terminal domains were found for AR, ER and PR [24]. Inter-domain communication also regulates ligand-independent transcriptional silencing: deletion of the PPARγ N-terminal domain prevents corepressor binding [25].

The A/B domains can be modified by phosphorylation and other post-translational, covalent modifications and confer distinct functional properties of nuclear receptors. In the case of ligand-activated receptors, AF-1 modifications have generally a tissue-specific modulatory effect on their transcriptional properties. For instance, the MR N-terminus harbors a serine/threonine-rich nuclear localization signal (NL0) that can be regulated by phosphorylation and influence receptor subcellular localization [26]. An elegant mechanism of regulation of the activity of RXR is provided by the piggyback nuclear exclusion of RXR upon association with Nur77, in a Nur77 AF-1 phosphorylation-dependent manner [27]. Similarly, MEK1-mediated phosphorylation of serine at position 84 inhibits PPARγ1 nuclear localization [28], although an alternative mechanism involving Pin1-mediated proteasomal degradation of PPARγ has been recently proposed [29]. Preventing phosphorylation at this residue in vivo generates mice with increased insulin sensitivity when fed a high fat diet [30]. Taken together, these and other data suggest that translocation of NRs to the nucleus is a property which can be very rapidly regulated by various signaling cascades.

Post-translational modifications also affect the intrinsic transactivating potential of NRs, i.e. by modulating their ability to recruit transcriptional comodulators, or by modifying the polypeptide half-life, both properties being in some instances intimately linked [31,32]. Very interestingly, phosphorylation of the A/B domain of GR by p38 MAPK was shown to induce stable tertiary structure formation in this domain, hence favoring its interaction with coregulatory proteins [33]. In turn, this tertiary structure may be stabilized by protein-protein interactions, as reported for the AR AF-1 [34]. More physiologically, the estrogenic effects of EGF are partially mediated by the phosphorylation of ER AF-1 by EGF-activated MAPKs [35]. In the case of orphan receptors, whose transcriptional activity is strongly dependent on AF-1 integrity, covalent modifications of this region have a very strong impact on their transcriptional output. Amino acid motifs in the A/B domain of Nurr1 mediating ERK5- or ERK2-mediated transcriptional activation have been identified [36,37]. Evidences for other post-translational modifications occurring in the N-terminus of NRs are scarce. Phosphorylation-dependent SUMOylation the AF-1 of ERRγ represses its transcriptional activity [38]. AR is SUMO-1ylated in its AF-1 domain at a SUMO consensus sequence found in all steroid receptors, thus inhibiting androgen-regulated signaling [39]. Conversely, N-terminal SUMO-1ylation of PPARγ strongly increases its transactivating potential [40]. However, as discussed below, SUMOylation in the C terminal AF-2 region is now viewed as a critical mechanism regulating the balance between transactivating and transrepressive functions of NRs.

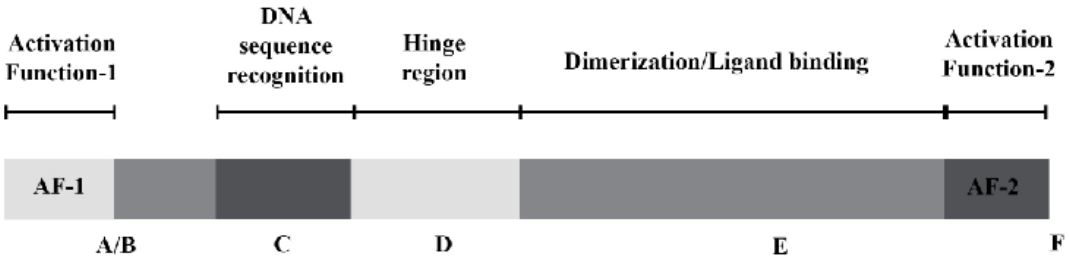

Figure 1. General structural organization of nuclear receptors. Letters from A to F represent nuclear receptor domains from N-terminus to C-terminus of the nuclear receptor respectively. The structure and functions of each domain is detailed in the text.

The DNA-binding domain

The DNA-binding domain (DBD) or C domain is the most conserved domain within the nuclear receptor family. Its main function is to recognize and bind specific DNA regulatory sites called response elements (REs) [41] The core DBD region contains about 66 amino acids, but many nuclear receptors additionally contain a less conserved C-terminus, a poorly structured motif of about 25 amino acids called the C-terminal extension region (CTE). As the CTE is located in the so-called hinge region, its features will be detailed in the corresponding paragraph.

The DBD is a highly structured, very compact globular domain composed by a pair of perpendicular α-helices stabilized by two C4 zinc-binding domains each coordinating tetrahedrally a zinc atom, a short β-sheet, and a few stretches of amino acids [42,43]. Each receptor monomer establish specific DNA contact through the first N-terminal helix (helix 1) which directly interacts with the major groove of the DNA half-site. A motif called the P box is critical for the DNA-binding specificity of the receptor [43-47]. Three amino acids of the α-helical P box distinguish nuclear receptors that will bind to the core AGAACA half-element (the “GSV-P box” initially found in GR) or to the AGGTCA half-element (the “EGG-P box” initially found in ER). Structural studies revealed that V and E amino acids make direct and unique contacts with the DNA half-site [48,49].

Nuclear receptor homo- or heterodimers establish contacts with two DNA half-sites that can be arranged in different geometry and separated by a spacer of varying length (see below and [50]). The C-terminal helix (helix 2) contributes to stabilization of the overall DBD structure, establishes weak, non-specific contact with DNA. A 5-amino acid loop defines a strong dimerization interface (D box) for homodimer formation and contributes, to a much lesser extent, to heterodimer stabilization [8,51-53].

DNA also provides a template for dimer assembly, which in turn induces conformational changes of the DNA double helix, most notably by inducing distortion of the minor groove to facilitate sequence recognition by the CTE [54]. This phenomenon is correlated with increased DNA bending in vitro, which has been documented for a number of nuclear receptors [55-58]. The relevance of this phenomenon when response elements are in a chromatinized environment is not clear however, although intrinsic DNA bendability affects GR binding to nucleosomal response elements in vitro [59]. The important role of nucleosome assembly and of histone post-translational modifications on the DNA binding affinity and transcriptional activity of nuclear receptors was demonstrated in vitro [60-63] and in vivo [64].

Although being a domain poorly accessible when receptor dimers are bound to nucleosomal DNA, the DBD can be the target of post-translational modifications. Much attention has been paid to kinase-mediated regulation of nuclear receptor affinity, and consequently a wealth of data document the generally inhibitor role of DBD phosphorylation. Indeed, as expected from the introduction of a repulsive charge, phosphorylation of the DBD of HNF4 [65,66], T3R [67] and ER [68] decreases their DNA binding activity. In a possibly related fashion, phosphorylation of a number of nuclear receptors in this region alters their nuclear retention and decreases their transcriptional activity [65,69,70]. In contrast, phosphorylation of TR2 [71] and of FXR [72] increased their DNA binding activity and interaction with PGC-1α respectively. Other covalent modifications such as RARα methylation or 15d-PGJ(2) adduct formation on ERα favor or inhibit receptor activity, respectively [73,74].

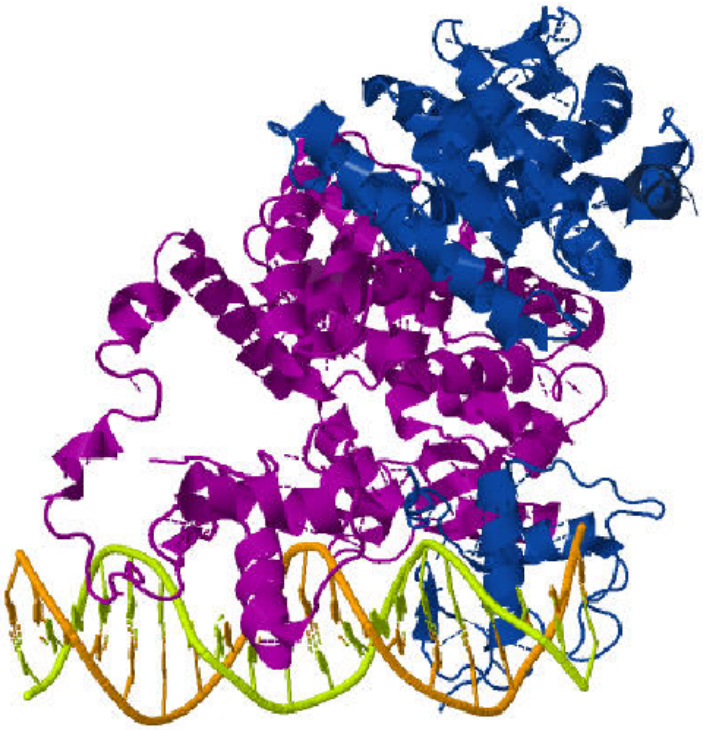

Figure 2. Crystal structure of PPARγ-RXRα complex bound to a DR-1 response element. Crystallographic coordinates were obtained from the RCSB protein databank (PDB 3E00) and visualized using the Jmol software. PPARγ is purple and RXRα is blue.

The hinge region

The flexible hinge, or D region, also called the C-terminal extension of the DBD (CTE) links the C-domain to the multifunctional C-terminal E/F ligand-binding domain and displays very low amino acid identity and similarity between nuclear receptors. Being located between two functionally and structurally important domains, it seems likely that its functions, deduced mostly from deletion and/or site-directed mutagenesis, may also reflect structural and functional alterations of these neighboring domains. Nevertheless, hinge regions of numerous nuclear receptors have been extensively dissected from a molecular point of view and shown to contain motifs responsible for regulating the subcellular distribution of nuclear receptors. Such a function has been demonstrated for ER [75], AR [76], VDR [77] and Dax1 [78] and reflect the presence of conserved nuclear localization sequences (NLS). The hinge region is also involved in tethering activities. The GR hinge region interacts with GR corepressors HEXIM1 and Bag-1 [79,80]. A natural variant (V227A) in the PPARα hinge region is associated with dyslipidemia and this mutation increases PPARα interaction with the nuclear corepressor NCoR [81]. Quite similarly, natural hinge variants of T3R display impaired dissociation of NCoR and recruitment of the coactivator SRC-1 upon agonist binding [82]. Supporting its role as a flexible link between the DBD and the C terminal LBD, hinge domain mutations affect the synergy between the AF-1 and AF-2 domains of ER [83]. Furthermore, the conserved 3D structure of receptor heterodimers, irrespective of the geometry of the bound DNA response element, highlights this physical property [12]. The hinge domain integrity is also conditioning the DNA binding affinity: in vitro assays showed that alternative splice variants affecting the hinge region sequence of FXR display distinct DNA binding affinities [84].

The CTE of monomeric receptor and of some dimeric receptors (also called T box and/or A box) adopts specific conformations which are context-dependent [85,86]. T and A boxes of dimeric receptors such as T3R, RARs, RXRs and VDR form an alpha-helical structure in solution and establish non-specific contacts with DNA [87-91] which can convert to an extended conformation favoring DNA binding in RXR homodimers and RXR-RAR heterodimers [92,93]. In contrast, the CTE of monomeric receptors such as rev-erbs, nur77 and ERRs establish specific contacts with DNA sequences located immediately 5' of the NR response element through the A box, which adopts an extended loop conformation [94-96]. The CTE is the major determinant of heterodimer polarity on half-site DNA [11].

The other function of CTE via the T-box is to provide an additional dimerization interface with the second zinc finger helix of RXR. Recent crystallographic analysis of PPAR-RXR-DNA complexes revealed a previously unknown dimerization interface between the RXR CTE and the PPARγ LBD [11], although the relevance of this structure has been challenged [12]. In contrast, the PPARγ CTE makes extensive DNA interaction by binding to the AAACT DNA sequence upstream of the core response element. The interaction with this 5' flanking sequence is similar to that observed with the Rev–Erb CTE [97,98].

As other domains, the hinge domain can be regulated by post-translational modifications such as methylation, acetylation, phosphorylation and sumoylation. p300-catalyzed acetylation of ERα hinge region regulates its transactivation properties and ligand sensitivity [99]. SUMOylation of RORalpha by both SUMO-1 and SUMO-2, as well as that of ERalpha has been reported, and mutations preventing SUMOylation generate transcription-defective

receptors [100,101]. In contrast, hPPARα SUMOylation on lysine 185 increases the selective recruitment of NCoR and decreased transcriptional activity [102]. Phosphorylation of serine residues in RARα, RORα4 and Nur77 are detrimental for receptor-mediated transactivation, either by decreasing DNA recognition or by preventing receptor dimerization [103], while phosphorylation of the PPARα hinge domain favors transactivation over tethered transrepression [104]. No investigations were carried out to identify structural changes induced by these covalent modifications, nor are reports describing when such modifications occur on such a sterically hindered environment.

The E or ligand-binding domain

As the domain accommodating lipophilic ligands capable of activating or repressing the transcriptional activities of nuclear receptors, it has attracted considerable interest as a paradigm for a transcriptional molecular switch, and as a target for synthetic analogs since these receptors control signaling pathways involved in a wide range of pathophysiological processes. Since the first crystallization of the RXR LBD [105], more than 600 3D structures related to nuclear receptor LBD structures have been reported, and about 3000 publications relate to some aspects of LBD structure and function. For more details, readers may refer to recent reviews of this fascinating field, linking 3D structure determination and modeling to pharmacology and therapeutics [51,106].

The E domain (or LBD) of nuclear receptors is a multi-functional unit comprising, in addition to the ligand binding pocket, homo- and heterodimerization interfaces and a comodulator binding region. The LBD acts as a molecular switch by interpreting the ligand structure into conformational changes which will convert the receptor in a transcriptional activator or repressor. Although the ligand has been long considered as the sole conformational modifier, it is now recognized that DNA response elements also induce structural transitions (see below). Nevertheless, the LBD remains the main architectural feature triggering biological responses to very diverse lipophilic molecules.

X-ray crystallography established the E domain as organized as a three-layered antiparallel α-helical sandwich composed by 12 α-helices, including a β-sheet (s1-s2) which is part of the ligand binding pocket (LBP). The LBP is located inside of this structure and is composed of a group of surrounding helices [51]. The LBP of nuclear receptors is a highly variable region, both in volume, ranging from 300 to 1500$Å^3$, and in structure. Such diversity allows the binding of a variety of molecules ranging from phospholipids to heme, including steroid and fatty acid derivatives and highlights the broad spectrum of physiological actions of nuclear receptors.

Ligand-LBP interactions involve amino acids located in most receptors in helices 3, 5 and 10/11. Additional interactions are brought into play as a function of the receptor and the chemical structure of the ligand. Hydrophobic interactions, hydrogen bonding networks and the steric size and shape of LBPs determine the strength and specificity of LBD-ligand complex [107]. This atomic network is variable according to receptor isoforms, allowing the design of isoform-selective agonists or antagonists [108].

Ligand binding causes conformational changes of nuclear receptors, which involve repositioning of H3, H4, L3-L4 and H12. Helix 12 (initially termed the AF-2 activating domain or AF-2 AD) is stabilized against the LBD core, generating a hydrophobic groove made of

helices 12, 3, 4 and 5. This structure allows the LBD to interact with the LXXLL signature motif found in most if not all reported primary nuclear receptor coactivators [109]. This interaction is further stabilized by a charge clamp made in most cases of a lysine in H3 and a glutamic acid in H12, which is required for optimal binding of coactivator molecules [110-113]. Subtle changes in ligand structure seem to affect the coactivator binding interface, providing a molecular basis for the varying efficacy and potency of nuclear receptor agonists [112]. In a more extreme fashion, antagonist binding positions helix 12 to cause a steric obstruction of the LXXLL binding groove. Importantly, the helix 12 region contains a degenerated LXXLL motif allowing for this interaction. Alternatively, antagonism can be exerted by generating a structure favoring the recruitment of corepressor molecules such as SMRT and NCoR or by preventing H12 proper folding. [113-117]. Intriguingly, some nuclear receptors act, in the absence of ligand, as transcriptional repressors. While it is acknowleged that this repressive action is physiologically important, the structural basis for this ligand-independent repression was unknown until recently. Two reports described a specific structure in RARα and rev-erb-α in the LBD that forms an anti-parallel β-sheet with corepressor amino acids, identifying a novel interaction interface [118,119] and documenting a structural basis for the mechanism of derepression, which necessitates the active removal of corepressor molecules. Finally, the LBD harbors a dimerization interface, the core of which mapping to H7, H9, H10, H11, loops L8-L9 and L9-L10. Although ligand binding has been long suspected to promote nuclear receptor dimerization [119-124], structural studies did not provide evidence for ligand-induced reshaping of this dimerization interface [106,125].

As other domains, the LBD is the target of posttranslational modifications. While it is beyond the scope of this review to provide an exhaustive list of identified covalent modifications (see also [126]), it is worth noting here SUMOylation plays an important role in channeling the transcriptional activity towards transactivation or tethered transrepression. SUMOylation of PPARγ at K365 is required for transrepression of the iNOS promoter in macrophages and targets PPARγ to the NCoR complex bound to NF-kappa-B regulated promoters [127]. This mechanism is detailed below. In an analogous manner, agonist-induced SUMOylation of LXRβ in the LBD promotes its interaction with GPS2 and binding to the NCoR complex associated to acute phase response genes [128]. PPARα also controls negatively hepatic gene expression in a sex-specific manner. Such a repression is exerted for example on Cyp7b1 expression, known to divert DHEA from the testosterone biosynthesis pathway. This occurs through the SUMOylation-dependent PPARα docking to the Cyp7b1 transactivating GA-binding protein, corepressor and HDAC recruitment to this promoter and DNMT3-catalyzed DNA methylation of a neighboring cis-activating SP-1 site [129]. Phosphorylatoin can exert opposite effects on NR activity through very diverse mechanisms. ATPase class 1 type 8B member [familial intrahepatic cholestasis 1 (FIC1) protein] activates FXR via PKCzeta-dependent phosphorylation of FXR at Thr-442. This covalent modification promotes the nuclear translocation of FXR and subsequent FXR target gene activation [130]. Through the combination of non genomic and genomic effects, retinoid acid activates the p38MAPK/MSK1 pathway, leading to phosphorylation of two serines in N-terminal domain and in RARα LBD and of histone H3. Phosphorylation of RARα increases the binding efficiency of cyclin H to the loop L8-L9 and promotes the right positioning of cdk7 and phosphorylation of RARα AF-1, to finally trigger RARα target genes activation [131]. This non-limitative set of examples thus point to the very complex integration of signaling events into nuclear receptor-mediated events.

The F domain

The F domain is located at the extreme C-terminus of NR. Because of its high variability in sequence, little is known about its structure and functional role. The length of the domain F can vary from no to 80 amino acids [132]. Crystal structure of progesterone receptor revealed that the F domain adopts an extended β-strand conformation [133] which may, in the case of RAR dimers, contact the dimerization partner [134]. Differences in ER isotype transcriptional activity are partly due to a variable F domain structure. Based on amino acid sequence, it is predicted that ERα F domain is an α-helical region followed by an extended β-strand-like region, separated by a random coil stretch. In contrast, ERβ domain F is more likely not to adopt an α-helical structure [135]. Mutagenesis and functional studies showed that domain F does not exert its activity independently and that it is dispensable for ligand binding or transcriptional activity. Nevertheless, deletion of the domain F or part of it may perturb NR activity and interactions with co-regulators. Deletion of the domain F eliminates the ability of human ERα to activate transcription via interaction with SP-1 [136]. HNF4, which harbors the longest domain F in its alternatively spliced isoform HNF4α2, is transcriptionally more active and is more responsive to overexpression of the co-activators NCoA2 and CBP [137]. The F domain of HNF4α1 interacts also with NCoR2/SMRT [138]. Interestingly, deletion of the F domain of RARα increased co-activator binding but decreased co-repressor binding [134]. Thus the F domain can be engaged in interactions with transcriptional co-regulators [139]. Moreover, different point mutations among domain F of ER suggested its involvement in ligand-receptor interaction, and impacts on the ligand responsiveness of ER tethered to an AP-1 response element [140]. Finally, the F domain can be covalently modified by phosphorylation and affect ER basal transcriptional activity. O-GlcNAcation of this domain leads to decreased ability of ER to bind to an estrogen response element in vitro [141].

DNA RESPONSE ELEMENTS GEOMETRY, ARCHITECTURE AND RECOGNITION BY NUCLEAR RECEPTORS

DNA sequence recognition and binding is the initial step of the transactivation process mediated by nuclear receptors. Consequently, NR monomers or dimers are positioned on RE which are made of one or two hexameric half-site motifs. Adopting a different geometry, they form palindromes, direct (DR), everted (ER) or inverted repeats (IR) separated by a spacer of varying length and sequence. Four conditions can be distinguished that determine the uniqueness of the response element. They are (i) the nucleotide sequence of the DNA-half sites, (ii) their relative orientation (iii) the sequence of the spacer and (iv) the length of the spacer.

Some NRs, mainly orphans, bind to DNA as monomers. The monomeric Nurr1 binds to a hormone response element 5'-AGGTCA-3' flanked by a 5' 1 to 6-bp long A/T-rich sequence [142]. This sequence referred to as an Nur77/NGFI-B response element (NBRE) [143] is also the target of Nur77 monomers [144]. Nurr1 can however dimerize with RXR, and in this configuration can display significant affinity for DR with spacing ranging from 10 to 27 bases [145]. A similar promiscuity in binding to naked DNA is observed for SF-1, FTZ-F1, rev-Erb-α and RORα which target a single copy of this extended core recognition sequence, although rev-Erb-α can also bind to a specific DR-2 RE [146,147].

Receptors binding to DNA as homodimers, exemplified by the steroid hormone receptors GR, MR, AR and PR recognize two consensus half-sites 5'-AGAACA-3' or in case of ER 5'-AGGTCA-3' arranged as inverted repeats spaced by 3 bp (IR3) [148]. Formation of stable head-to-head homodimers is dependent on discrete dimerization interfaces located in both the DBD and the LBD (Figure 3).

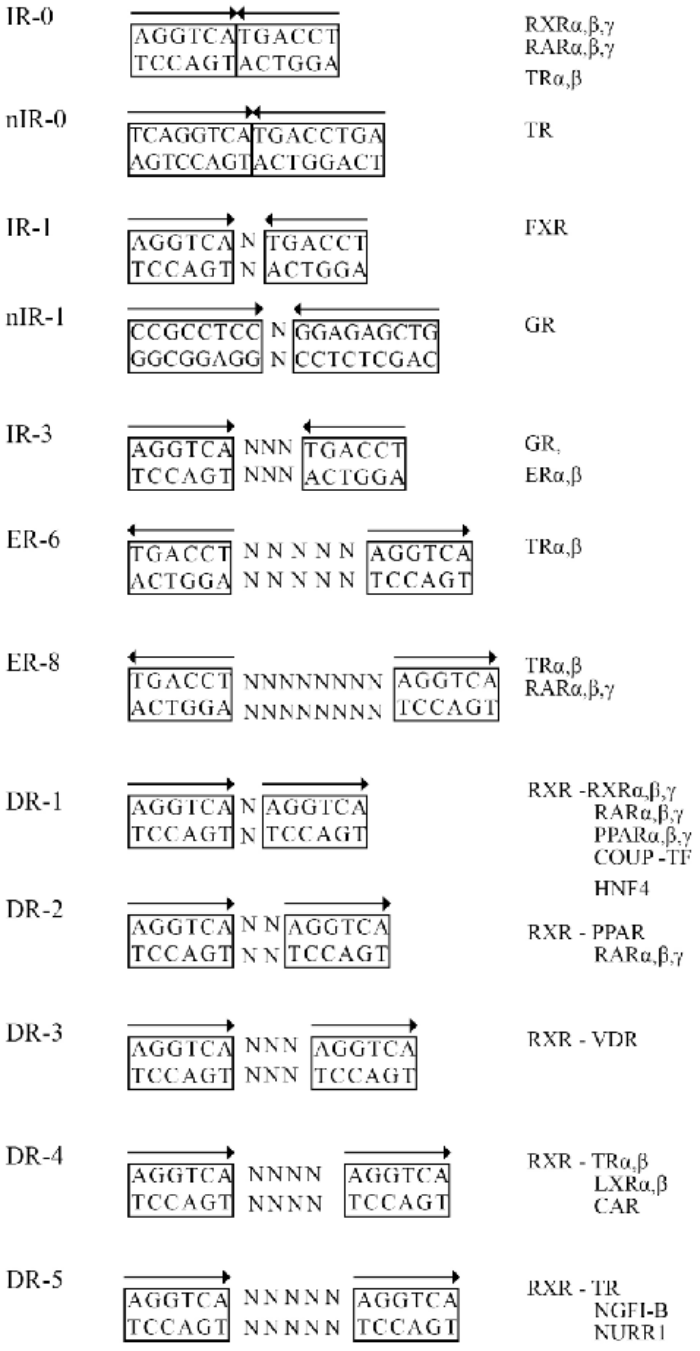

Figure 3. Different architecture of selected response elements of nuclear receptors. IR - inverted repeat, ER - everted repeat, DR - direct repeat, 'N' indicates any nucleotide, “n” indicates negative response elements.

Nuclear receptors that form heterodimers with RXRs recognize REs composed of two half-site motifs arranged as direct (DR), inverted (IR) or everted repeats (ER), the core consensus sequence being 5'-AGGTCA-3'. For instance PPARs, RARs, VDR and T3R

recognize direct repeats following a specificity rule called the 1-2-3-4-5 rule [149-151]. Some RXR partners display a more relaxed specificity: PXR can bind to a variety of DNA response elements with various spacing, which includes direct repeats DR-3, DR-4, and DR-5, and everted repeats ER-6 and ER-8 [152,153]. FXR prefers binding to an inverted repeat of the ideal sequence 5'-AGGTCA-3' separated by 1 bp (IR-1) [154], but several different response elements have been reported, including ER8 [155] and DR1 [156].

RXR partners can be divided into two groups depending on their functionality as heterodimers. Permissive RXR-containing heterodimers can be activated by RXR agonists in the absence of the agonist for the RXR partner. This group includes PPAR, LXR and FXR. Nonpermissive heterodimers formed by RXR and RAR, TR, VDR cannot be activated by RXR agonists and require agonists of the RXR partner to be activated [157-159].

Heterodimers can adopt various polarities when bound to different REs, and RXR can be positioned either upstream or downstream of the heterodimer partner. This relative orientation and its impact on the transcriptional activity of receptors has been dissected for RAR-RXR heterodimers. On DR2 and DR5 elements, RXR occupies the 5’ hexameric motif, whereas the RAR partner occupies the 3’ motif. The polarity is reversed on DR1 response elements. This structural arrangement has dramatic consequences on the transactivation properties of RXR-RAR heterodimers, as RAR agonists are unable to activate transcription from a DR1 RE. This relates to the allosteric control of NCoR assembly on these various DR REs [157,160,161] whose geometry imposes an important structural adaptation of receptor domains. In support of this, DNA binding of RXR-VDR dimers was shown to alter VDR H12 structure [125]. Crystallographic structures of isolated GR DBD bound to DNA identified the so-called “lever arm”, located between the two GR zinc fingers, which adopts different conformations according to the RE geometry and influences coactivator recruitment [162]. Other heterodimers such as PPARα-RXRα bind to DNA similarly to RAR-RXRα and form a polar head-to-tail interaction with DR1, where RXRα binds exclusively to the 3' site [11, 92]. For VDR assembled on a DR3, TR and LXR on a DR4 and NGFI-B on a NBRE, the RXR DBD was found to bind to the 5' upstream half-site [50,89,163].

Thus several structural features are brought into play to limit nuclear receptor DNA binding promiscuity, in addition to tissue- and cell-specific expression and limited ligand availability. It is worth noting that these rules have been defined using naked DNA templates. However, a genome-wide bioinformatic search for any of these consensus sequences will yield at least a hit every 500-1000 bp. This number is at odds with the number of actual NR binding sites determined by Chip-seq experiments (several thousands for ER and PPARγ, [164-167]) and the number of regulated genes determined in similar conditions (a few hundreds). Moreover, many of these sequences are located very distal to the transcriptional start site (TSS) when considering a linear sequence, either 5' or 3' to the TSS. Chromosomal conformational studies revealed that enhancer sequences act in cis with respect to promoter sequences, implying chromatin looping between TSS and enhancer sequences [165,168,169]. Quite intriguingly, the functionality of such an association is characterized by the induction of the so-called enhancer-templated non-coding RNA (eRNA) emanating from the distal binding site [170], a phenomenon whose functional significance has not yet been elucidated but which is not restricted to NR-mediated transcriptional control [171]. Genome-wide mapping of nuclear receptor binding sites also revealed the statistically- and biologically-significant association of a fraction of REs with other transcription factor binding sites. This led to the identification of cell-specific “pioneering factors” such as FoxA1, which act by priming NR

DNA binding sites to bind their cognate NRs [172,173]. There are thus multiple mechanisms controlling the association of NRs with DNA, all of them having a significant impact on the assembly of NRs on chromatin templates and productive recruitment of the transcription machinery.

GENERAL MECHANISMS OF TRANSCRIPTIONAL REGULATION BY NUCLEAR RECEPTORS

As already mentioned above, nuclear receptors can control transcriptional events by exerting either a positive, direct effect or by imposing a repressed state to regulated promoters. They can also mediate, through protein-protein interaction, a repressive effect on a variety of other signaling pathways under the control of transcription factors such as AP-1, NF-kappa-B or C/EBP. Each of these aspects will be described below to provide a global view of the most recent concepts which have emerged in the field in the past years (Figure 4).

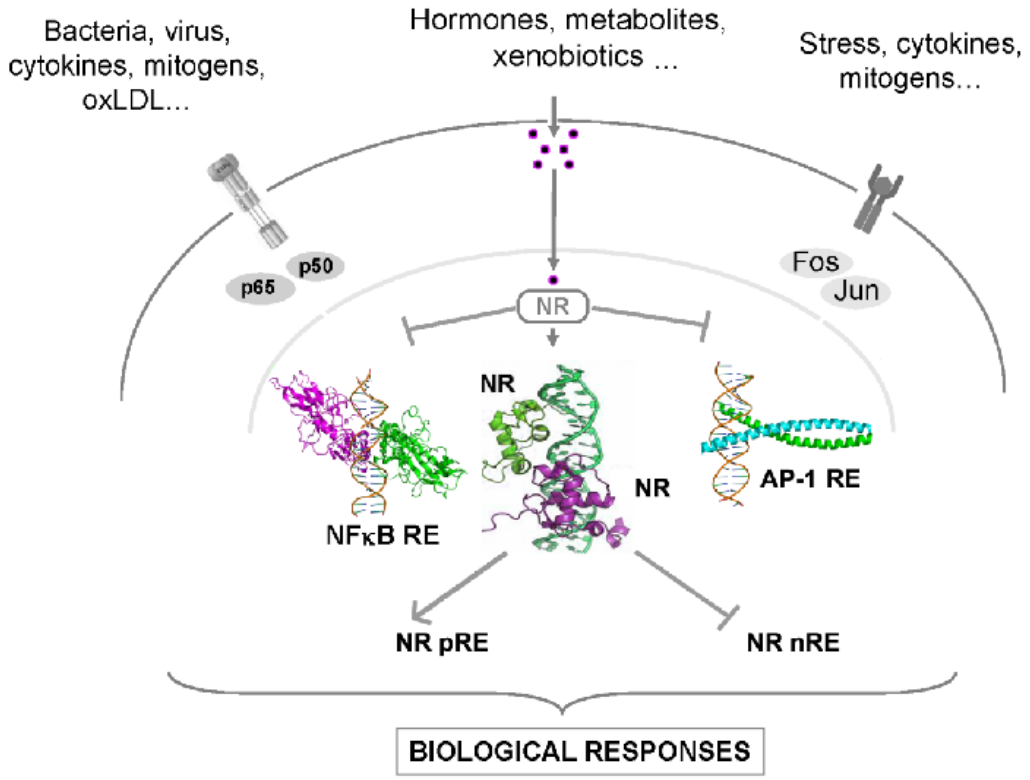

Figure 4. General mechanism of NR action. Nuclear receptors may act in two different ways. Upon ligand binding nuclear receptors forming heterodimers with RXR interact with a specific positive gene response element (pRE) and activate mRNA transcription of target genes. Alternatively, they may interact directly with repressive, negative response elements (nRE). The major suppressive effect of nuclear receptors is however thought to be mediated by monomers interaction with subunits of AP-1 and NF-kB transcription factors, and hamper the expression of inflammatory-related genes (see text for details).

Transcriptional activation

An important feature of steroid hormone receptors and of most of the heterodimeric nuclear receptors is the ability to activate transcription of target genes upon ligand binding. In general, this mechanism comprises ligand-dependent conformational changes of the nuclear receptor associated to chromatinized REs, that trigger co-repressor complex release and the sequential recruitment of co-activator complexes that modify chromatin structure and promote the assembly of the transcription initiation complex at regulated promoters. Various co-activators were identified for NRs and the repertoire is specific for certain cell types, genes and signals. Thus binding of agonists stimulates the exchange of co-repressors for co-activators necessary for transcriptional activation. Of note, the ligand-dependent association of NR corepressors such as LCoR and RIP140, through LXXLL motifs may play a significant role in transcription attenuation [174,175], however this mechanism has not been studied in great detail and will not be discussed here.

Nuclear receptor corepressor binding

In the unliganded state, NRs are associated to corepressor complexes. These complexes are composed of a subunit (SMRT/NCoR2 or NCoR1) directly interacting with the receptor through a degenerated LXXLL motif, which harbor a consensus sequence L/I-X-X-I/V-I or LXXXI/LXXXI/L also called the CoRNR box [176,177]. This CoRNR box motif interacts, as the coactivator LXXLL motif, with amino acids from the LBD hydrophobic groove. This interaction interface is remodeled upon agonist binding and helix 12 positioning occludes part of the CoR binding interface. As mentioned above, additional CoR binding interfaces, as well as novel CoRNR boxes have been described [118,119,178], suggesting the use of alternative mechanisms for NR-corepressor interaction. Corepressor complexes are built around the SMRT or NCoR subunits, which harbor a conserved repression domain on which the core repressive machinery (including HDAC3, GPS2 and TBL1 or TBLR1) is assembled. Recent structural and functional studies highlighted a central role for TBL1 in assembling this very large complex (ca. 1-2 MDa) [179]. In some cases, ligand-binding is sufficient to inhibit co-repressor recruitment (e.g. for RXR and TR), but more generally the active removal of the co-repressor complex is required. This points again to the critical role of TBL1/TBLR1 which encompass a F-box domain interacting with the ubiquitin-conjugating enzyme H5 (UBCH5) and a 19S-proteasome complex, which mediates ubiquitination and proteosomal degradation of SMRT- or NCoR-GPS2-HDAC3 complexes [180].

Nuclear receptor coactivator binding

Since the seminal discovery of SRC-1/NCoA1 as a progesterone receptor coactivator [181], more than 350 coactivators have been identified so far. This prodigious amount of polypeptides exhibit various enzymatic activities involved in the regulation of histone modification and chromatin remodeling, initiation of transcription, elongation of RNA transcripts, mRNA splicing and elongation, and proteasomal termination of nuclear receptor complexes. Their involvement and relative activity in nuclear receptor-controlled processes is modulated by their cell-specific expression levels and post-translational modifications, conditions which have been reviewed recently [182,183]. It is also nowadays accepted that

many of these coregulators participate in molecular events driven by other transcription factors.

The coactivator family has been divided in two subfamilies. The first one defines coactivators which interact directly with NR AF-1&2 regions such as the SRC coactivators, CBP and p300. The second one includes other proteins which interact with primary coactivators such as CARM1, CoCoA, Fli-I... Primary and secondary coactivators are recruited to regulated promoters in an orchestrated fashion [184]. Since this issue is devoted to nuclear receptors involved in metabolism control, only coactivators associated to such an activity will be briefly described here.

The p160 and p300 families: Co-activators belonging to the p160 family [NCoA1/SRC-1, NCoA2/TIF2 (known as SRC-2 or GRIP1) and NCoA3/RAC3 (also known as SRC-3, ACTR, pCIP or TRAM-1)], p300 and the cAMP response element-binding protein (CBP) bind to the NR LBD via an alpha-helical LXXLL motif [185,186]. Co-activators such as CBP and p300 posses histone acetylase transferase (HAT) activity, which has a critical role in regulating NR-mediated transcription [187]. N-terminal tail acetylation of histone H4, which is likely to establish contacts with the histone H2A/H2B dimer, prevents this interaction and destabilizes chromatin compaction. Additionally, acetylation weakens the interaction of the histone tails with DNA [188]. Consequently, the chromatin is decondensated allowing the promoter initiation complex to bind at the promoter site.

Data emerging from studies of knockout animals suggest that the SRCs play critical and distinct roles in controlling energy homeostasis. SRC-1-/- mice have decreased energy expenditure and are prone to obesity. In opposition, SRC-2-/- mice are protected against high-fat diet-induced obesity, but can lead to a condition reminiscent of a glycogen storage disease type 1a. The ablation of SRC-3 generates mice highly resistant to high-fat diet-induced obesity. Collectively, these data and others point to a complex, but critical role of SRCs in metabolic regulation which has been in most instances related to the control of PPARγ transcriptional activity [189]. However, given the pleiotropic role of SRCs, it is very likely that other mechanisms contribute to these metabolic effects.

The ATP-dependent remodelling complex SWI/SNF: the SWI/SNF complex has a role in metabolic control, as it was identified in yeast to be essential for mating-type switching and growth on sucrose. The SWI/SNF family is evolutionary conserved and plays an important role in ATP-dependent chromatin remodeling [190] by catalyzing the disruption of DNA-histone interactions and sliding of the nucleosome along DNA [191]. The human homolog BAF complex is a multimeric entity of 1.2 MDa including BRG1/hBRM, BAF polypeptides (BAF155/170, BAF60, BAF57, BAF53a/b, BAF47, BAF250a/b, BAF200, BAF45a/b/c/ d, Brd9, and Brd7) and actin. Several of these subunits harbor LXXLL motifs and have been identified not only as nuclear receptors coactivators for ER [192,193], AR [194], RAR [193,195], FXR [196] and GR [197], but also as corepressors of SHP [198], as SWI/SNF components can be integrated in corepressor complexes [199]. Interestingly, the BAF60a subunit displays a circadian expression in mouse liver and, acting as a coregulator of RORα, regulates the expression of clock and metabolic genes [200].

The mediator complex: Like the SWI/SNF complex, the Mediator complex has been originally identified in yeast and subsequently characterized in other eukaryotic cells. A number of

studies described its role as a catalyzer of the transcription preinitiation complex (PIC) assembly at activated promoters. Through direct interaction with RNA polymerase II, general transcription factors (TFIID, TFIIH) and elongation factors, Mediator plays a key role in RNA polymerase II-controlled transcription [201]. Investigations about the role of Mediator in NR research gained momentum when it was realized that Mediator-like complexes bind directly to NRs [202-206]. Mediator is organized in four structural modules and includes more than 20 subunits, of which the Med1 subunit contains LXXLL motifs [207]. The liver-specific Med1 KO induces hepatic steatosis in a PPARγ-dependent manner [208], in agreement with its adipogenic [209] and PPARγ coactivator roles [210]. Skeletal muscle-specific KO of Med1 enhances insulin sensitivity and improves glucose tolerance and confers resistance to high-fat diet-induced obesity [211]. Thus given its broad and key roles in transcriptional regulation through a direct interaction with RNA polymerase II, Mediator is viewed as being the last complex recruited cyclically to NR-regulated promoters [184].

Transcriptional repression

Transcriptional repression by unliganded receptors

Some nuclear receptors can actively repress transcription in the absence of ligand. This process is related to the recruitment of co-repressor complexes. There are several co-repressor complexes characterized, but the most commonly studied complex comprises nuclear receptor co-repressor (NCoR), silencing mediator for retinoid and thyroid hormone receptors (SMRT), histone deacetylase 3 (HDAC3), transducin-α-like 1 (TBL1), TBL-1-like related protein (TBLR1) and G-protein-pathway suppressor 2 (GPS2) [212,213]. HDACs posses a well-characterized role in transcriptional repression by deacetylating N-terminal lysines of histone proteins thus generating a condensed, transcriptional inactive chromatin structure. It was reported that SMRT and NCoR contain a deacetylase-activating domain which can trigger the enzymatic activity of HDAC3 [214].

In addition, other corepressor complexes have been described, such as SWI/SNF-containing complexes as mentioned above, PRC1&2 and CoRest complexes. Like the NCoR/SMRT complex, tethering these multiprotein entities to promoters leads to histone and DNA covalent modifications, followed by chromatin compaction and/or DNA masking. A critical step in NR-mediated transcriptional activation is the dismissal of corepressor complex from the DNA-bound receptor. In vitro assays have demonstrated that agonist-induced conformational changes are sufficient for SMRT or NCoR dissociation from the receptor, in agreement with crystal structure data. However, dynamic models of de-repression involving post-translational modifications of corepressor complex subunits leading either to their nuclear exclusion and/or degradation have been described [215]. The mechanism(s) by which such an active derepression takes place is as of yet unknown.

Direct transrepression by liganded receptors

Ligand-bound NRs repress the transcription of some genes by a mechanism called negative regulation. This process occurs with multiple NRs and genes and was detailed for GR and TR. It has been suggested that these NRs recognize and bind negative response elements and downregulate specific target genes. The analysis of specific DNA sites revealed that

negative glucocorticoid response elements (nGRE) and negative thyroid response elements (nTRE) are different from positive response element that mediates transcriptional activation [216,217]. Overlapping binding sites for transcription factors such as Oct-1/Pbx, AP-1 and SP1 were found for negative response elements of GR and TR, and found to dictate the transcriptional cis effect of the response element [218-221]. These data thus posit that negative cis-acting glucocorticoid response elements exert such an activity by interacting with other transcription factors. However, a recent report described a novel class of negative glucocorticoid REs, organized as inverted repeats with a 1bp spacer, on which glucocorticoids promote the recruitment of GR-corepressor complexes [222]. Such a mechanistic principle does not seem to hold true for T3-mediated transcriptional repression. As detailed for the αTSH gene, the corepressor SMRT is recruited to the nTRE and promotes histone deacetylation. Upon agonist treatment, SMRT dismissal is correlated with histone acetylation and gene repression [223,224]. Furthermore, functional studies have shown a role for SRC-1 in transcriptional repression mediated by liganded TR [225,226]. The mechanistic basis for such a reversal of transcriptional activity is not known, but could be mediated by post-translational modifications such as phosphorylation, acetylation or SUMOylation of promoter-associated histones and/or of coregulatory proteins [227-229]. Thus direct repression occurs via distinct mechanisms which are receptor- and context-dependent. These studies also pinpoints to the versatility of coregulator complexes, which may exert either positive or negative effects on the transcriptional outcome following NR agonist stimulation.

Tethered transrepression by liganded receptors

The mechanism referred to as tethered transrepression engages negative crosstalk of ligand-activated nuclear receptors with other signal-dependent transcription factors, including NF-kappa-B and activator protein-1 (AP-1). This process modulates inflammation in various cells of the central nervous system, the immune system as well as the liver, etc and interferes with cellular proliferation in various tissues.

Several mechanisms can be proposed to account for such a repression: (i) repression of PIC assembly on NF-kappa- or AP-1 regulated promoters [127,128,230,231]; (ii) inhibition of RNA polymerase II conversion towards an elongation-competent form [232,233]; (iii) upregulation of the expression of the inhibitor of NK-kappa-B [234]; (iv) interaction with upstream components of the NF-kappa-B or AP-1 activating cascade [235-237]; (v) coactivator exclusion by competition [238,239] and (vi) direct physical interaction with AP-1 or NF-kappa-B (mostly p65) subunits [240-243], although this process is much more complex and requires multiple factors in living cells [244].

Interestingly, inflammatory programs triggered by TLR-3, 4 or 9 activation in macrophages are only partially inhibited by GR, LXR and PPARγ agonists, each receptor inhibiting about one-third to one-half of the induced genes. Intriguingly, inhibited clusters of genes by each receptor were only partially overlapping [238].

NR co-repressors such as NCoR and SMRT play an important role in ligand-dependent tethered transrepression. NCoR-deficient macrophages display a derepressed expression of various AP-1 and NF-kappa-B-related genes, an effect linked to NCoR (or SMRT, [245]) association to these DNA-bound transcription factors [246]. Much like NR-mediated

transcription, activation of signaling pathways leads to the transcription of NF-kappa-B-driven genes by removal of the corepressor complex through a proteasome-dependent pathway. NR activation upon ligand binding promotes tethering of sumoylated NR to NF-kappa-B complexes, which interrupts corepressor complexes clearance, hence maintaining the promoter in a repressed state [127]. More recently, sumoylated LXRs were found to be targeted at transrepressed promoters through interaction with a NCoR complex component, coronin A. This interaction prevents corepressor turnover by preventing oligomeric actin recruitment [247]. This very elaborate process has been described for LXR.in mouse macrophages, whereas transrepression of the acute phase reaction (APR) in mouse liver by LXR involves GPS2 rather than coronin A [128,247]. Thus, as suspected from many previous studies, tethered transrepression follows different mechanistic schemes which are receptor-, gene- and cell type-specific.

The structural features of NR specifically involved in transrepression are not clearly defined. Extensive mutagenesis studies of T3R, RAR, PPARγ, GR and ER (see for examples [239,248-261]) did not yield a clear-cut and unifying model for tethered transrepression. Taken as a whole, it clearly appeared that coactivator recruitment through the AF-2 domain is not required for this activity, as well as direct DNA binding. There are also strong evidences suggesting that homo- or hetero dimerization is not mandatory [239,262]. The lack of well-defined molecular structures involved in transrepression is an important pitfall in designing screening methods aiming at identifying dissociated ligands which would preferentially elicit tethered transrepression in inflammatory diseases.

NUCLEAR RECEPTORS AND NON-GENOMIC SIGNALING PATHWAYS

NR ligands regulate gene expression by genomic actions which are described above. Nevertheless, NR ligands also exhibit non-genomic effects manifested by the rapid and transient activation of several kinase cascades, which can be attributable to a subpopulation of NRs located at the cell membrane, although this point is still debated. Accordingly, conserved palmyltoylation sites have been identified in GR and ER [263-265], and together with MR, these receptors have been detected in lipid rafts [265-268].

This extranuclear localization provides a mean for steroid receptors to interact with various kinases. Estrogens trigger protein-protein interaction between ER and Src/p21ras/Erk and PI3K/Akt, through the SH2 domain of c-Src and the regulatory subunit of PI3K respectively. Estrogen-mediated induction of these kinase cascades plays an important role in cell proliferation in breast cancer and vascular function [269-273]. Progestins can induce the Src/Erk1/2 pathway mediated by the interaction of two domains of the progesterone receptor (PR) with the LBD of ER. This crosstalk is essential for progestin induction of DNA synthesis and cell proliferation in breast cancer [274]. A complex of activated PR, ERK and its target kinase Msk1 is recruited to the promoter after hormone treatment and phosphorylates serine 10 of histone H3, where it induces the recruitment of SRC-1, RNA polymerase II and chromatin remodeling complex (hSnf2h and Brg1). This example constitutes a link between kinase cascade activation in the cytoplasm, chromatin remodeling, and transcriptional activation in the nucleus [275] which is possibly conserved for retinoid receptors [276]. It has been suggested that aldosterone can counteract vasoconstriction via stimulation of endothelial NO production. This occurs through a mechanism which engages PI3 kinase and its interaction with MR [277]. A similar mechanism seems to underlie the decreased vascular

inflammation and reduced myocardial infarct size following ischemia and reperfusion injury induced by glucocorticoids [278].

Recent evidences show that dexamethasone, a synthetic GR agonist, reduces cPLA2 activation which releases arachidonic acid. This mechanism seems to be glucocorticoid receptor-dependent but transcription-independent [279,280]. Plasma membrane-bound GR [281] has indeed been described in a variety of cell types [282,283] and GR has been shown to associate to Src in lipid rafts [268].

Non-genomic effect events similar to those described for steroid hormones occur for retinoids. It has been reported that RAR is present in the cytoplasm and in membranes where it associates with PI3K or Src [284]. Retinoic acid (RA) rapidly activates mitogen-activated protein kinases (MAPKs) such as ERK and p38MAPK in fibroblasts, mouse embryocarcinoma cells, mammary breast tumor cells and leukemia cells [131,285,286]. A novel unexpected non-genomic activity has been demonstrated for RARα: RARα is transported to neuronal dendrites where associates with glutamate receptor 1 (GluR1) mRNA, via its C-terminal F region and, as a result, inhibit the translation of this mRNA. RA binding abrogates this translational repression. These effects have been correlated to the regulation of synaptic functions and neuronal plasticity controlled by RA [287-289].

Non-genomic effects were also observed for nuclear receptor ligands involved in metabolic control. Although there is no evidence for membrane-bound PPARγ, the synthetic agonist rosiglitazone (RGZ) as well as the natural agonist 15ΔPGJ2 regulate glucose and lipid metabolism and sperm activation in human spermatozoa by a rapid mechanism involving protein phosphorylation [290]. In human microvascular endothelial cells, RGZ interferes with pro-inflammatory actions of TNF and IFNγ by direct inhibition of ERK1/2 phosphorylation in a PPARγ-dependent manner [291]. RGZ-mediated ERK1/2 regulation and PI3K inhibition was observed in human adrenocortical cells and PC3 prostate cells [292,293]. Conversely, in vascular smooth muscle cells 15ΔPGJ2 and TZD activated the MEK/ERK pathway via PI3K [294]. Importantly, the energy-sensitive AMP kinase is activated by TZD-stimulated PPARγ, inducing acetyl CoA carboxylase phosphorylation, stimulation of glucose uptake and fatty acid oxidation in skeletal muscle, liver and adipose tissue [295,296].

Thus non genomic effects of NR ligands, mediated or not by an extranuclear subpopulation of NRs introduce a new layer of complexity in NR biology which must be determined when studying biological and pharmacological effects of NR ligand administration. Although impaired by technical limitations, the study of the subcellular localization of NRs in pathophysiological conditions may help deciphering mechanisms controlling the broad spectrum of biological responses controlled by NRs. Worth noting, the mitochondrial effects of some NRs such as SHP [297], GR [298] and Nur77 [299-301] which play an important role in apoptosis regulation through protein-protein interaction, deserve further investigations for other members of the NR family.

CONCLUSION

NRs are modular transcription involved in multiple pathophysiological processes. They can be viewed as an assembly platform on chromatin for multimeric coregulators which will dictate the cell-specific and even gene-selective transcriptional ouput of target cells. In

addition to direct ligand binding, these multi-proteic complexes are integration modules of other signaling pathways which can additionally adjust NR-driven promoters response to their extracellular cues. With the advent of high throughput genomic, epigenetic and proteomic techniques, a NR system biology can now be elaborated to bring a global and detailed view of NR contribution to human biology and diseases.

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Chapter 3

MOLECULAR MECHANISM OF PPARα ACTION AND ITS IMPACT ON LIPID METABOLISM AND INFLAMMATION

ABSTRACT

Peroxisome Proliferator-Activated Receptor alpha (PPARα/NR1C1) is a ligand-activated transcription factor belonging, together with PPARγ (NR1C3) and PPARβ/δ (NR1C2), to the NR1C subfamily of the nuclear receptor superfamily. PPARα adopts the classical tertiary structure of nuclear receptors, with a C-terminal ligand binding domain (LBD) and a central, highly conserved DNA-binding domain (DBD) allowing interaction between PPARα and specific DNA sequence elements termed Peroxisome Proliferator Response Elements (PPRE). This architecture allows the formation of transcriptionally active heterodimers with the Retinoic X Receptors (RXRs) thought to play a major role in PPARα-mediated transactivation. Naturally occurring PPARα agonists are eicosanoids, fatty acids (FAs) and prostaglandin derivatives. A major biological function of PPARα is the control of lipid utilization in tissues with high oxidative energy rates such as skeletal muscle, heart and liver. Genome-wide studies in rodents indicate that the vast majority of PPARα target genes is involved in peroxisomal and mitochondrial fatty acid transport and oxidation. Furthermore, PPARα maintains glucose homeostasis and promotes ketone body synthesis upon food deprivation. PPARα also exerts pleiotropic anti-inflammatory effects in hepatic and vascular inflammation, by repressing pro-inflammatory cytokine and acute phase gene expression levels mainly via interference with the AP-1 and NFκB signaling pathways. In clinical practice, fibrates, which are synthetic PPARα agonists, are widely used as lipid-lowering drugs in the treatment of dyslipidemia. Recently, PPARα activation, especially when combined with PPARβ/δ agonism, has been found to improve steatosis, inflammation and fibrosis in rodent models of non-alcoholic steatohepatitis (NASH), thus representing a new potential therapeutic area.

Keywords: PPAR/β-oxidation/inflammation/transrepression/liver/NASH

INTRODUCTION

Peroxisome Proliferator-Activated Receptor alpha (PPARα/NR1C1) is a ligand-activated nuclear receptor highly expressed in the liver, initially identified as the molecular target of xenobiotics inducing peroxisome proliferation in rodents [1]. Beside PPARα, the PPAR subfamily contains two other isotypes encoded by different genes, PPARβ/δ (NR1C2) and PPARγ (NR1C3), each displaying isoform-specific tissue distribution patterns and cellular functions [2, 3]. In rodents and humans, PPARα expression is enriched in tissues with high fatty acid oxidation rates such as liver, heart, skeletal muscle, brown adipose tissue and kidney [4, 5], although it is present in virtually every tissue and cell type including the intestine, vascular endothelium, smooth muscle cells [6-8] and immune cells such as monocytes, macrophages and lymphocytes [9, 10]. PPARα is a nutritional sensor which allows adaptation of the rates of fatty acid catabolism, lipogenesis and ketone body synthesis in response to feeding and starvation. PPARα was identified as a transcriptional master regulator of genes involved in peroxisomal and mitochondrial β-oxidation, fatty acid transport and hepatic glucose production, the latter likely being rodent-specific [11]. Multiple physiological conditions such as stress, starvation, hormones, including growth hormone, glucocorticoids, insulin and leptin, can modulate PPARα expression and activity [12, 13]. In rodents, PPARα negatively regulates pro-inflammatory and acute phase response signalling pathways in models of systemic inflammation, atherosclerosis and non-alcoholic steatohepatitis (NASH) [14-16]. A wide range of structurally different PPARα ligands were identified including endogenous fatty acids and fatty acid derivatives such as eicosanoids and leukotrienes [17]. Certain herbicides, pesticides and plasticizers can also function as PPARα ligands, identifying PPARα, at least in rodents, as a promiscuous xenobiotic receptor [18]. Fibrates are PPARα agonists used in clinical practice since the early 1960s, many decades before PPARs were discovered [19]. In patients with dyslipidemia, fibrates lower plasma triglyceride levels and small dense LDL particles, and raise HDL-C levels. In prevention studies fibrates reduce major cardiovascular events, especially in patients with high triglyceride and low HDL-C plasma levels [20-22]. In type 2 diabetes mellitus (T2DM) patients, fibrates lower the need for laser-intervention in retinopathy, lower-extremity amputations and possibly nephropathy, suggesting that fibrates can protect against microvascular complications [23-25].

In this chapter, we present our current understanding of the transcriptional activation and repression mechanisms of action of PPARα, the spectrum of target genes and chromatin binding maps from recent genome-wide studies, and pay particular attention to PPARα-triggered fatty acid turnover and anti-inflammatory PPARα activities in the liver. The role of PPARα, in conjunction with PPARs in non-alcoholic steatohepatitis (NASH) will be elaborated based on available pre-clinical data.

FUNCTIONAL ANALYSIS OF PPARα STRUCTURE

The human and mouse PPARα genes localized on chromosome 22 and chromosome 15 respectively, encode a polypeptide of 468 amino acids with strong inter-species homology (91%). In both species, the coding DNA sequence (CDS) spans the 3' region of exon 3, exons 4-7 and the 5' extremity of exon 8 [1, 26, 27]. Several transcript variants have been described for the PPARα gene including an alternatively spliced exon within the 5-

untranslated region (5'-UTR) of the human PPARα gene [28] and transcripts encoding truncated, dominant negative isoforms in man, swine and jerboa [29, 30].

Canonical structure of PPARα

PPARα has a canonical nuclear receptor organization with five domains starting from the N-terminal A/B to the F domain at the C terminus. Domain-specific functions can be distinguished based on mutagenesis studies and comparative analysis (Table 1). In a native conformation, these domains integrate various intracellular signals to control the transcriptional activity of multiple target genes. The A/B domain contains the activation function-1 (AF-1) region providing basal, ligand-binding independent activity as well as ligand-dependent activity, which can be potentiated by MAPK-dependent phosphorylation of serines 6, 12 and 21 [31]. Moreover, comparative studies of chimeric PPARα/β/γ protein identified the N terminal AF-1 region as a determinant of isotype-specific target gene activation [32, 33]. The A/B domain is connected to the structurally conserved DNA-binding domain (DBD), harboring a two zinc-finger structure, able to bind PPREs, localized in gene regulatory regions and organized as direct repeats of two hexamer core sequences AGG(A/T)CA separated by one nucleotide (DR-1). Evidence has emerged that PPARα/β/γ bind PPREs uniquely as a heterodimer with Retinoic X Receptors (RXRα/β/γ) [34]. The A/T rich motif upstream of the DR-1 provides a polarization signal of the PPAR-RXR heterodimer, and may confer isotype binding specificity. Accordingly, PPARs interact with 5'-extended hexamers, whereas RXR binds to the downstream motif of the response element [32]. The hinge region (domain D) is a highly flexible region linking the DBD (domain C) and the ligand binding domain (LBD). The structural integrity of the hinge region conditions the interaction of PPARα with nuclear receptor corepressors, such as NCoR, in the unliganded conformation [35, 36]. The hinge region is a target for posttranslational modifications, such as phosphorylation catalyzed by protein kinase C (PKC) on serines 179 and 230. SUMOylation also targets the hinge domain of human PPARα at lysine 185 and potentiates NCoR recruitment [37, 38].

	Domain		PTM	Function
N-term	**A/B**	**AF-1**	MAPK-dependent phosphorylation at Ser 6, 12 and 21	Ligand-dependent/independent activation function Target gene specificity
	C	**DBD**		Binding to PPRE Interaction with cJun
	D	**Hinge region**	PKC-dependent phosphorylation at Ser 179 and 230 SUMOylation at lysine 185	Providing NR structure flexibility Potentiating NCoR recruitment
C-term	**E/F**	**LBD/AF-2**	SUMOylation at lysine 358	Ligand binding specificity Interaction with RXR and p65 Interactions with multiple co-regulators e.g. CBP/p300 and SRC/p160

Table 1. Functional analysis of PPARα structural domains. PPARα displays a classical NR canonical architecture. PPARα domains (from A to F) fulfil distinct functions by providing interaction surfaces with other TFs, co-regulators and ligands, thus contributing to specific PPARα transcriptional

regulation. PPARα is subjected to several post-translational modifications (PTM) that markedly impact receptor function (details in the text).

The C terminal LBD is the only domain of PPARα whose structure has been solved by X-ray crystallography [39]. Similar to PPARγ and PPARβ/δ, the PPARα LBD is composed of a helical sandwich flanking a four-stranded β-sheet and contains the activation function-2 (AF-2) helix. The volume of 1400 Å3 for the PPARα ligand binding pocket (LBP) is only slightly different than the total volume reported for PPARγ and PPARβ/δ LBPs of 1600 and 1300 Å3, respectively [40, 41]. Nevertheless, the PPARα LBP is more lipophilic and less solvent-exposed than the LBPs of the two other PPAR isotypes, hence allowing the binding of more saturated fatty acids. In contrast to PPARγ, the PPARα AF-2 helix is more tightly packed against the LBD core when complexed with an agonist [42]. Tyrosine 314 within the PPARα pocket has been identified by crystallography as the main determinant of isotype selectivity of different classes of ligands [39]. The PPAR isotypes exhibit 60-70% identity between their LBDs, explaining the difficulty of designing isotype-specific ligands [39]. The AF-2 domain undergoes conformational changes in a ligand-dependent mode, thereby directing various co-activators such as CBP/p300 and SRC-1, carrying LXXLL motifs (L – leucine, X – any amino acid), to a hydrophobic cleft on the PPARα LBD surface, thus promoting the formation of an active transcriptional complex. The AF-2 domain may also fulfil an essential role in ligand-dependent repression of certain genes. It has been suggested that agonist binding unmasks lysine 358 in the LBD for SUMOylation, hence conferring repressive activity to PPARα [43].

Endogenous and synthetic PPARα agonists

Evidence has emerged that natural PPARα ligands are fatty acid derivatives formed during lipolysis, lipogenesis or fatty acid catabolism. Certain substrates of the first rate-limiting enzyme of peroxisomal β-oxidation, acyl-CoA oxidase (ACOX), have been hypothesized to be PPARα agonists. Consistently, disruption of ACOX results in increased peroxisome proliferation, hepatocarcinoma and elevated transcription of PPARα target genes [44, 45]. Endogenous eicosanoid derivatives, including the chemoattractant leukotriene B4 (LTB4) and 8(S)-hydroxyeicosatetraenoic acid (8(S)-HETE), the murine 8-lipoxygenase (8-LOX) product from arachidonic acid, are thought to be endogenous PPARα agonists. Nevertheless, their physiological importance has not been fully established [46]. The oxidized phospholipid fraction of oxidized low-density lipoproteins (oxLDL) enhances transcriptional activity of PPARα and induces its target gene, fatty acid transport protein-1 (FATP-1) in human primary endothelial cells [47]. Liver-specific inactivation of fatty acid synthase (FAS), an enzyme catalysing the synthesis of fatty acids, allowed the identification of endogenous PPARα activators. Liver FAS knockout in mice fed a fat-depleted diet develop hypoglycemia and liver steatosis, which can be reversed by dietary fat and Wy14,643, a synthetic PPARα agonist. This suggests that products of FAS-dependent de novo lipogenesis, in contrast to circulating free FA deriving from peripheral tissues, serve as PPARα activators [48]. Moreover, mass spectrometry analysis of PPARα isolated from liver of FAS knockout mice revealed the presence of 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (16:0/18:1-GPC) bound to its LBD, thereby identifying this phospholipid as an endogenous PPARα ligand [49]. In line, adipose triglyceride lipase (ATGL)-dependent hydrolysis of hepatic and cardiac muscle intracellular TG yields lipid ligands for PPARα activation [50, 51]. Moreover, heart-

specific lipoprotein lipase (Lpl)-knockout mice are protected against cardiomyopathy provoked by chronic activation of PPARα in transgenic mice with cardiac-specific PPARα overexpression (MHC-PPARα mice), thereby suggesting that TG-derived products of LPL hydrolysis serve as PPARα activators in heart [52]. In clinical practice, fibrates, synthetic PPARα agonists, such as gemfibrozil, fenofibrate and ciprofibrate, are used in the treatment of primary hypertriglyceridemia or mixed dyslipidemia as observed in T2DM and the metabolic syndrome [19].

MECHANISM OF PPARα-DEPENDENT TRANSACTIVATION

Formation of transcriptionally active multiprotein PPARα complexes

Ligand-activated PPARα recruits numerous co-activator proteins including members of the CBP/p300 and SRC/p160 family which exhibit histone acetyltransferase (HAT) activity and other co-activators forming the transcriptionally active PPARα-interacting cofactor (PRIC) complex [53]. Using a protein microarray approach, we found that PPARα can physically interact with a battery of co-regulators including CBP, SRC-1, PGC-1α and MED-1 in a ligand-dependent manner. Those interactions were not see with a truncated PPARα deleted in its AF-2 domain [our unpublished data]. Disruption of the PPAR-binding protein (Pbp)/mediator subunit 1 (Med1) gene encoding an anchor component of the mediator transcription complex showed its essential role in PPARα-dependent gene regulation. PBP/MED1 stabilizes and directs a large transcription initiation complex containing numerous co-activators and RNA polymerase II to the DNA-bound PPAR-RXR heterodimer (Figure 1a) [54, 55]. As previously mentioned, RXR is required for PPAR binding to PPREs in vitro. Both PPAR and RXR agonists enhance PPAR action on its target genes suggesting a cooperative crosstalk between these two nuclear receptors [56, 57]. However, RXR homodimers may specifically bind DR-1 PPREs independent of PPARα, and induce the transcriptional action of PPARα target genes through a co-activator-dependent mechanism [58].

Genome-wide transcriptomic and PPARα chromatin binding maps

Genome-wide localization and activity-occupancy studies revealed that induction of PPARα target gene expression by PPARα agonists is associated with increased binding of PPARα to chromatin, rather by strengthening affinity and stability of existing interactions, than creating de novo ligand-inducible binding regions. Interestingly, almost half of the PPARα-binding regions in human hepatoma cells are located within introns, whereas only 26% of them are localized in close vicinity (<2.5 kb) of the transcription start site (TSS) [59]. This binding profile corresponds to the one reported for other nuclear receptors including PPARγ [60]. In addition, genome-wide profiling of LXR, RXR and PPARα in mouse liver shows overlapping regions in chromatin binding LXR-RXR and PPARα-RXR heterodimers. Nevertheless, only a few percent of LXR and PPARα binding sites contain consensus DR-4 and DR-1 elements, respectively [61]. Indeed, de novo motif analysis identified the co-enrichment of PPARα binding regions in CCAAT-enhancer-binding protein α (C/EBPα) and TATA binding protein (TBP) motifs [59]. This is in line with previous studies reporting the existence of “hot spots” at open chromatin regions favouring NR binding to degenerate motif sequences by mechanisms engaging protein-protein interactions between multiple transcription factors such as GR, RXR, TBP, STAT and C/EBP family members [62].

MODELS OF PPARα TRANSCRIPTIONAL REPRESSION

Besides its ability to positively regulate gene expression, PPARα, similar to other members of the nuclear receptor superfamily, can act as a negative regulator of transcription in a mechanism referred to as transrepression. Several models for NR-driven transcriptional repression have been proposed, emphasizing multiple and complex mechanisms of action.

PPRE-independent transcriptional repression

Evidence has emerged that PPARα negatively regulates pro-inflammatory signalling pathways via protein-protein interactions. The so-called tethering mechanism has been extensively evaluated in vitro and in mouse models of acute inflammation. Ligand-activated PPARα represses cytokine-induced Il-6 gene expression via interference with AP-1 and NFκB pro-inflammatory signalling pathways. PPARα-driven transrepression involves direct physical interactions between PPARα, the p65 Rel homology domain and the N-terminus JNK-responsive part of cJun (Figure 1c) [15]. Moreover, enhanced synergistic transrepression of NFκB-driven gene expression was observed upon simultaneous activation of PPARα and GR, a well-characterized repressor of NFκB signalling pathways (Figure 1d) [63]. However, PPARα and GR transrepress distinct, but overlapping sets of genes in vascular endothelial cells [64]. PPARα activation down-regulates hepatic acute phase response genes, such as fibrinogen, serum amyloid A (Saa) and haptoglobin (Hg) in rodent, and C-reactive protein (CRP) gene expression in human hepatocytes.

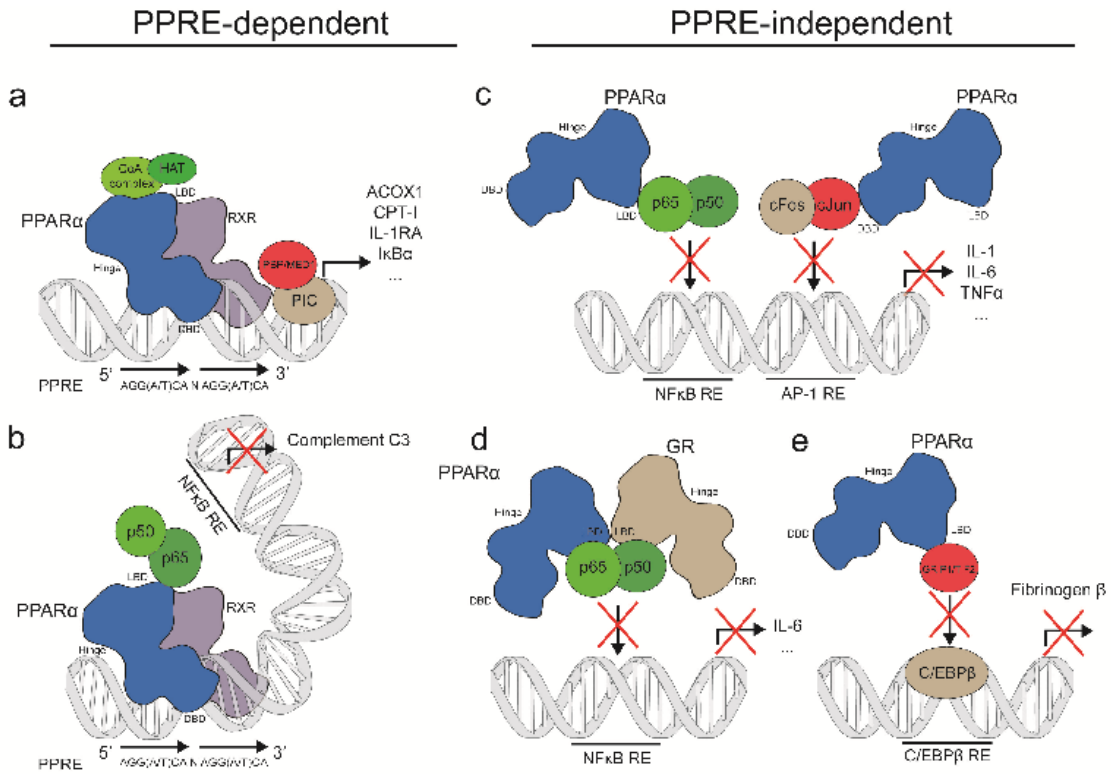

Figure 1. Models of PPARα transcriptional regulation. Several models of PPARα transcriptional regulation have been proposed via which PPARα modulates expression of its target genes as well as

pro-inflammatory transcription factors and acute phase response genes. a. formation of the PPRE-dependent ligand-activated transcriptional complex containing PPARα-RXR heterodimer, co-activators, HAT, PBP/MED1 and the transcriptional preinitiation complex (PIC). b. PPRE-dependent inhibition of NFκB transcriptional activity. Upon ligand activation DNA-bound PPARα directly interacts with p65 to abolish its binding to a NFκB response element (NRE) in the complement C3 promoter. c. PPARα directly interacts with pro-inflammatory transcription factors cJun and p65 to negatively regulate their target genes by a mechanism that is thought to be PPRE-independent. d. Simultaneous ligand-activation of GR and PPARα leads to the enhanced repression of TNF-induced IL-6 transcriptional activity by the mechanism that stems from a direct GR-PPARα physical interaction. e. PPARα downregulates fibrinogen β transcriptional activity via ligand-dependent mechanisms engaging physical interaction between PPARα and GRIP-1/TIF-2.

Mechanistically, PPARα down-regulates mRNA expression and protein levels of GP80 and GP130, which are components of the IL6-receptor, thus disrupting the STAT3 and cJun signalling pathways involved in the acute phase response [14]. Similarly, in liver, fibrates down-regulate IL-6-stimulated fibrinogen β expression via PPARα-dependent titration of glucocorticoid receptor-interacting protein-1/ transcription intermediary factor-2 (GRIP-1/TIF-2) thus interfering with C/EBP β activity (Figure 1e) [65]. Another mechanism of PPARα-dependent transcriptional repression has been identified in the control of ERR-driven mitochondrial respiration and cardiac contraction. In certain conditions such as pressure overload in the heart or during fasting, the PPARα-SIRT1 complex binds directly to a single hexad ERRE motif, thus mediating downregulation of ERR target genes by competition, in a RXR-independent manner [66, 67].

Studies on PPARγ, LXR and LRH-1-mediated transrepression identified a co-repressor-based anti-inflammatory mechanism in mouse macrophages and liver [68-70]. Transcriptional co-repressors, such as NCOR and SMRT, serve as active repressors of pro-inflammatory target genes through the recruitment of histone deacetylases (HDACs) [70]. Ligand-dependent SUMOylation targets NRs to pro-inflammatory gene promoters, thus preventing the clearance of NCOR/HDAC complexes by the ubiquitylation/19S proteosome machinery [71]. A specific SUMOylation site has been identified for PPARα [38]. Further studies should address the importance of posttranslational modifications and co-repressor interactions in PPARα-driven transrepression.

PPRE-dependent transcriptional repression

Recently, a novel PPRE-dependent model of transcriptional regulation has been proposed through a negative cross-talk between PPARα and p65 to diminish complement C3 promoter transcriptional activity in a human hepatoma cell line. Ligand-dependent activation of PPARα inhibits TNF-mediated up-regulation of complement C3 through the physical interaction between PPRE-bound PPARα and p65, to abolish p65 binding to the upstream NFκB response element (NRE) on the complement C3 promoter (Figure 1b) [72]. In line, genome-wide studies revealed the presence of STAT-PPAR binding motifs within ligand-inducible PPARα binding regions of significantly down-regulated genes. This suggests a direct negative crosstalk between PPRE-bound PPARα and pro-inflammatory transcription factors [59].

REGULATION OF FATTY ACID METABOLISM BY PPARα

PPARα-regulated cellular FA uptake

Fatty acids are transported into cells by membrane-associated fatty acid transport proteins (FATPs) [73]. FATP1, which catalyses ATP-dependent esterification of long chain fatty acids (LCFAs) and very long chain fatty acids (VCFAs) to their acyl-CoA derivatives, was identified as a direct PPARα target gene [74, 75]. Another plasma membrane FA transporter, fatty acid translocase (FAT/CD36), is positively regulated by PPARα ligands [76]. Interestingly, FATP1 and FAT/CD36 can translocate to mitochondria, thus directing intracellular fatty acid transfer [77, 78].

FA activation occurs through enzymatic acylation by the long-chain fatty acyl-CoA synthetases (LCAS/ACSL), localized on intracellular membranes. PPARα triggers Lcas gene transcriptional activity, hence promoting long-chain acyl-CoA formation for further use in oxidation [73, 79, 80]. Intracellular lipid trafficking is maintained by cytoplasmic polypeptides such as fatty-acid-binding proteins (FABPs) and acyl-CoA-binding protein (ACBP), which bind and transfer LCFA and LCFA-CoA to different organelles. In accordance, liver fatty-acid-binding protein (L-Fabp/Fabp-1)-deficiency in mouse primary hepatocytes leads to impaired LCFA nuclear distribution [81]. Functional PPREs have been identified within the promoters of the liver L-Fabp and the intronic region of Acbp genes [82-84]. Direct protein-protein interaction has been reported between PPARα and L-FABP, suggesting that L-FABP may channel PPARα ligands to the receptor [85, 86]. Consistently, a positive correlation between L-FABP protein concentration and PPRE-driven gene transcriptional activity was observed in HepG2 cells treated with synthetic PPARα agonists as well as saturated and polyunsaturated fatty acids (SFAs and PUFAs, respectively) [87].

PPARα and regulation of β-oxidation pathways

β-oxidation in peroxisomes and mitochondria encompasses several steps including FA uptake, dehydrogenation, hydration, further dehydrogenation and thiolytic cleavage. Nevertheless, different enzymes, encoded by separate genes, are involved in these processes in mitochondria and peroxisomes. From a physiological point of view, the differences between peroxisomal and mitochondrial β-oxidation are reflected in substrate specificity. Peroxisomes carry out the initial shortening of saturated and unsaturated very long-chain fatty acids, and branched chain FAs, whereas the majority of long-chain fatty acids is oxidized in mitochondria.

In rodents, PPARα promotes peroxisomal fatty acid uptake through the ligand-dependent regulation of peroxisomal membrane ATP-binding cassette sub-family D member 2 and 3 (ABCD2 and ABCD3) [88]. In rodents and primates, fatty acid transport across the mitochondrial membrane is triggered by a PPRE-dependent regulation of carnitine palmitoyltranserase I and II (CPT-I and CPT-II), localized in the outer and inner mitochondrial membrane, respectively [89-91]. Furthermore, in the mouse, PPARα controls transcriptional activity of malonyl-CoA decarboxylase in heart and skeletal muscle thus depleting malonyl-CoA, the natural inhibitor of CPT-I activity. In line, alterations in cardiac energy metabolism in Pparα-deficient mice are associated with decreased FAO and elevated levels of cardiac malonyl-CoA [92]. PPARα controls the gene expression levels of the rate-limiting enzymes of peroxisomal β-oxidation, including acyl-CoA oxidase 1 (Acox1), a flavoenzyme generating

enoyl-CoA and hydrogen peroxide [93]. Livers from Acox1-deficient mice reveal extensive microvesicular steatohepatitis and elevated PPARα target gene levels, leading to hepatocellular regeneration and massive peroxisome proliferation as a consequence of sustained activation of PPARα [44, 94, 95]. Enoyl-CoA hydratation to hydroxyacyl-CoA and its subsequent NAD(+)-dependent dehydrogenation are carried out by a single protein, known as the L-bifunctional enzyme (L-PBE/BIEN/EHHADH), which is highly inducible by PPARα via PPRE-dependent transactivation [96-101]. Subsequent cleavage of ketoacyl-CoA to acetyl-CoA and a two carbons shortened acyl-CoA molecule is catalyzed by thiolases, including the PPARα-dependent 3-ketoacyl-CoA thiolase (thiolase B). Acyl-CoA can then be hydrolyzed into free fatty acids and CoA by long chain acyl-CoA thioesterases, amongst which peroxisomal acyl-CoA thioesterase 2 (PTE-2) is a PPARα target gene in rodents [102, 103].

Since peroxisomes are unable to completely oxidize FAs, the full oxidation of shortened FAs takes place in the mitochondria. Mitochondrial β-oxidation is a highly effective process generating ATP through the electron transport chain. In the human hepatoma HepG2 cell line, PPARα regulates the critical reaction of mitochondrial β-oxidation by directly controling of medium-chain acyl-CoA dehydrogenase (MCAD) transcriptional activity [104]. The constitutive expression of very long chain acyl-CoA dehydrogenase (VLCAD) and long chain acyl-CoA dehydrogenase (LCAD) is reduced in livers of Pparα-deficient mice [105]. MCAD expression is elevated in heart and livers of fasted wild-type mice along with increased PPARα expression and activity, whereas Wy,14643 significantly increases hepatic expression of VLCAD, LCAD, MCAD, SCAD in wilde-type, but not in Pparα-deficient mice [105, 106].

PPARα and ketogenesis

During prolonged fasting, hepatic FAO rates increase, yielding acetyl-CoA units which are further converted into ketone bodies. Ligand-activated PPARα upregulates mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS), a rate limiting enzyme of ketogenesis which catalyses condensation of acetyl-CoA and acetoacetyl-CoA to generate HMG-CoA and CoA [107]. The mild phenotype of Pparα-deficient mice fed ad libitum becomes more pronounced during fasting, and is characterized by impaired FAO, lipid accumulation in liver and heart as well as hypoglycemia and inability to augment ketone body synthesis [12, 108]. Moreover, high-fat, low-carbohydrate ketogenic diet (KD)-fed mice show increased hepatic mRNA expression and plasma levels of the fibroblast growth factor 21 (FGF21), in parallel with the induction of PPARα [109]. Moreover, KD-fed mice in which Fgf21 was knocked-down reveal impaired hepatic expression of genes of FAO (Acox1, Cpt-I) and ketogenesis (Hmgcs, Bdh), indicating that FGF21 is required for the normal activation of these metabolic pathways [109]. Further studies identified FGF21 as a direct PPARα target gene, induced, in mouse and humans, in response to fasting and upon PPARα ligand administration [109, 110].

PPARα IN THE REGULATION OF HEPATIC LIPOGENESIS AND PLASMA LIPOPROTEIN METABOLISM

Molecular insights into the lipid normalizing effects of PPARα

Pharmacological activation of PPARα by fibrates reduces plasma TG and pro-atherogenic small-dense LDL-cholesterol (LDL-C) levels, and raises HDL-cholesterol (HDL-C) levels in individuals with mixed dyslipidemia [111-114]. Mechanistic studies in rodent models revealed that the reduction of plasma triglyceride-rich lipoprotein (TRL) levels upon PPARα activation is related to enhanced FA uptake, its conversion to acyl-CoA derivatives and further catabolism via the β-oxidation pathways. Moreover, the TG-lowering action of PPARα is also due its ability to increase lipolysis via induction of lipoprotein lipase (LPL) activity which catalyses the hydrolysis of lipoprotein TGs into free FA and monoacylglycerol. PPARα controls LPL mRNA expression through binding to a functional PPRE present in the human and mouse LPL gene promoters [115]. Furthermore, PPARα enhances LPL activity indirectly by decreasing mRNA levels and secretion of hepatic apolipoprotein (APO)-CIII, an LPL inhibitor [116]. Interestingly, glucose induces APO-CIII transcription in hepatocytes through a mechanism involving the transcription factors carbohydrate response element–binding protein (CHREBP) and hepatocyte nuclear factor-4 (HNF-4) [117]. Conversely, hepatic expression of APO-CIII is inhibited by insulin through insulin-dependent phosphorylation of forkhead box O1 (FOXO1), resulting in its displacement from the nucleus and inability to drive APO-CIII transcriptional activity [118]. In hepatocytes, inhibition of APO-CIII transcription by fibrates is the consequence of multiple cooperative mechanisms including PPARα-driven displacement of HNF-4 from the APO-CIII promoter, inhibition of FOXO1 activation of APO-CIII transcription via the insulin-responsive element (IRE) and inhibition of glucose-stimulated APO-CIII expression [116, 119].

In humans, fibrates increase plasma HDL-C by stimulating the synthesis of its major apolipoprotein constituents, APO-AI and APO-AII. However, species-differences exist between humans and rodents with respect to apolipoprotein regulation by PPARα. A functional PPRE is present in the human, but not rodent APO-AI promoter, as illustrated by increased human APO-AI production in humanized Apo-AI transgenic mice upon treatment with fibrates [120]. In contrast, APO-AI and HDL-C levels are elevated in Pparα-deficient mice and fibrate treatment leads to decreased Apo-AI mRNA in wild-type animals [121, 122]. In human and mouse liver, APO-AII expression is induced by PPARα. Hepatic human APO-AII gene transcription is induced by PPARα through its interaction with a PPRE localized within the APO-AII promoter region. A functional PPRE could not be identified within the mouse Apo-AII promoter [123], however, based on available data from the genome wide PPARα binding map [61], we inspected promoter regions of hepatic mouse Apo-AII for the presence of PPARα chromatin immunoprecipitation-sequencing (ChIP-seq) peaks and identified a PPARα binding peak also in the mouse Apo-AII proximal promoter, 100 base pairs downstream of the transcription start site (TSS) [our unpublished data]. Similarly, species-specific transcriptional regulation modes are observed for APO-AV, which enhances LPL activity, by PPARα [124, 125]. Several studies using human LPL transgenic/Apo-AV-deficient mice and human APO-AV transgenic/Lpl-deficient mice support the hypothesis that APO-AV reduces TG levels by trafficking VLDL and chylomicrons to proteoglycan-bound LPL for lipolysis [126, 127]. Several in vitro and in vivo studies in wild-type mice versus transgenic humanized APO-AV mice revealed that human, but not mouse APO-AV expression levels

are elevated in liver upon PPARα agonist administration [128, 129]. These findings are consistent with the identification of a functional PPRE in the human APO-AV promoter, whereas this region is non-functional in the mouse Apo-AV promoter [128, 129]. In humans, rare single-nucleotide polymorphic (SNP) variants in the APO-AV promoter region are associated with paradoxical decreases in plasma HDL-C and APO-AI in response to fibrates, whereas SNPs within the APO-AV gene are associated with enhanced lipid response to fibrate and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor (statins) therapy [130-133]. Thus, paradoxical responses to fibrate treatment in some individuals may be due to genetic variations in PPARα target geses, such as APO-AV.

PPARα and hepatic lipogenesis

Besides its ability to orchestrate lipoprotein metabolism, PPARα directly or indirectly controls lipogenic pathways in the liver. Lipogenesis is the metabolic pathway allowing FA synthesis when dietary carbohydrates are abundant. Dietary regulation of hepatic lipogenic genes is under control of the insulin-dependent transcription factor sterol regulatory element binding protein 1c (SREBP-1c) and the glucose-activated CHREBP [134]. In reporter gene assays, PPARα agonists enhance human SREBP-1c transcriptional activity, through direct PPARα interaction with a DR-1 element localized in the human SREBP-1c promoter. Consistently, PPARα binding to the human SREBP-1c promoter was demonstrated in vitro and in vivo, in human primary hepatocytes [135]. In turn, in mouse liver, the SREBP-1c target genes fatty acid synthase (Fas), acetyl-CoA carboxylase (Acc1) and stearoyl-CoA desaturase (Scd-1) are positively regulated by PPARα agonists [136-138]. Nevertheless, neither SREBP-1c nor its downstream targets have not been identified as direct PPARα target genes in the mouse, with the exception of Scd-1, for which a PPRE has been found within its promoter regulatory region [138]. In mice, fibrates increase the protein levels of the mature hepatic form of SREBP-1c by increasing the rate of proteolytic cleavage of its membrane-bound precursor form without changing Srebp-1c mRNA levels [139]. The insulin-dependent enhancement of SREBP-1c transcription requires the participation of Liver X receptors (LXR) and SREBP-1c itself [140, 141]. Moreover, via LXR-binding sites in the human and mouse Srebp-1c promoter, LXR agonists induce its transcriptional activity [135, 142]. PPARα can also indirectly modulate SREBP-1c transcription via cross-regulation of the LXR signaling pathway. In the mouse, PPARα appears required for the LXRα-dependent response of SCD-1 and FAS to insulin in re-fed conditions, suggesting a potential role for PPARα in the synthesis of endogenous LXRα ligands [143]. In human primary hepatocytes, PPARα agonists, cooperatively with insulin and LXR agonists, induce lipogenic genes, such as FAS and ACC1 [135].

In the mouse, PPARα controls the expression of FA elongases, such as elongase-6 (ELOVL-6) and elongase-5 (ELOVL-5) and FA desaturases (Δ5, Δ6 and Δ9 desaturases), which are involved in the synthesis of arachidonic acid (AA/ 20:4n-6), eicosapentanoic (EPA/20:5n-3) and docosahexanoic (DHA/22:6-n) acids from shorter PUFA, such as linoleic acid (LA/C18:2n-6) and α-linolenic acid (ALA/C18:3n-3) [144]. Hepatic expression of ELOVL-5 and ELOVL-6 as well as the Δ5, Δ6 and Δ9 desaturases is upregulated by PPARα agonism in wild-type, but not in Pparα-deficient mice [144, 145]. In line, hepatic levels of linoleic and α-linolenic acid are elevated in Pparα-deficient mice, due to the reduction of Δ6 desaturase expression [146].

Physiologically, PPARα coordinates different pathways of de novo lipid synthesis in fed state to supply storage of hepatic TG for periods of starvation. During fasting when the organism switches to the utilization of FA deriving either from the liver or from peripheral tissues, PPARα also shifts its activity to promote FA uptake and β-oxidation thus yielding substrates for ketone body synthesis to provide energy for peripheral tissues.

PPARα IN ACUTE AND CHRONIC LIVER INFLAMMATION

The metabolic syndrome (MS) is a constellation of risk factors often occurring together and predisposing to the development of cardiovascular diseases (CVDs), T2DM and non-alcoholic fatty liver disease (NAFLD). These risk factors (central obesity, insulin resistance, dyslipidemia and hypertension) are provoked by lifestyle factors, including physical inactivity and high-caloric diets (high carbohydrates, high saturated FA) interacting with genetic factors [147, 148]. Various components of the MS are associated with increased inflammation, characterized by high plasma levels of acute phase proteins (CRP, SAA) and cytokines, including tumor necrosis factor (TNF) and IL-6 [149, 150]. Moreover, the MS predisposes patients to atherosclerosis and NASH, diseases with a chronic inflammatory component. In vivo and in vitro studies provided evidence that PPARα counteracts atherogenesis and steatohepatitis progression likely due to its pleiotropic effects on lipid metabolism and inflammation.

PPARα and acute hepatic inflammation

The acute phase response (APR) is an orchestrated response of the organism to infection, trauma and tissue injury, in order to promote repair processes and restore physiological functions. The liver plays a crucial role in the APR by producing various cytokines, such as IL-1, IL-6 and TNF, which then trigger the synthesis of acute phase proteins (APPs), including the previously mentioned SAA, CRP as well as fibrinogen, haptoglobin (HG), α-2 macroglobulin and others [151]. Nevertheless, excessive or prolonged action of cytokines is potentially harmful and contributes to chronic inflammatory diseases.

In different mouse models of systemic inflammation, PPARα was shown to exert anti-inflammatory activities. PPARα activation with fenofibrate attenuates the IL-6-induced acute phase response in vitro and in vivo, by downregulating hepatic expression levels of Saa, Hg and fibrinogens α, β and γ in wild-type, but not in Pparα-deficient mice [14]. Similar inhibitory effects of PPARα agonists on IL-1β and IL-6-induced acute phase response were observed in mice with liver-restricted Pparα expression [152]. By contrast, treatment with IL-1β decreases expression of liver PPARα and its target genes, suggesting a negative cross-talk between IL-1β-induced inflammation and hepatic FAO regulation [153]. In line, lipopolysaccharide (LPS)-induced APR was counteracted by fibrates in Pparα-deficient mice with liver-specific reconstituted Pparα [152]. Interestingly, pretreatment with a PPARα agonist markedly prevented the LPS-induced increase of circulating IL-1, IL-6, and TNF, and the expression of adhesion molecules, such as ICAM-1 and VCAM-1 in the aorta, suggesting that liver PPARα controls, in a yet undefined manner, the systemic inflammatory response [152].

The anti-inflammatory effects of hepatic PPARα may also derive from its ability to up-regulate anti-inflammatory genes, such as the IL-1 receptor antagonist (Il-1ra) and IκBα, a

cytoplasmic inhibitor of NFκB, raising a possibility of cooperation between PPARα-dependent transactivation and transrepression to turn on anti-inflammatory pathways (Figure 1a) [154, 155].

PPARα action in non-alcoholic fatty liver disease (NAFLD)

NAFLD is a common cause of chronic liver disease which affects 10-24% of the population and is associated with insulin resistance and the MS [156]. The pathology initiates with hepatic steatosis, which in some individuals progresses toward non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis and finally liver failure. Transition from steatosis to steatohepatitis is mediated by increased lipid peroxidation and generation of reactive oxygen species (ROS) as well as of cytotoxic aldehyde products of lipid oxidation that initiate hepatic inflammation [157]. Lipidomic analysis of livers from patients with NASH revealed the presence of increased free cholesterol (FC) and an increased PUFA n-6 to n-3 ratio, mainly due to reduced n-3 PUFA [156, 158].

The ability of PPARα to counteract different stages of NAFLD has been studied in animal models which partially replicate the human pathology [159]. Administration of a methionine and choline-deficient (MCD) diet leads to the development of steatohepatitis in rodents, histologically similar to human NASH. Nevertheless, the MCD diet does not induce insulin resistance normally observed in human NASH. Pparα-deficiency in MCD-fed mice provokes more severe steatosis and hepatitis [16]. In wild-type mice, PPARα agonism normalizes histological changes due to its ability to prevent intrahepatic lipid accumulation, liver inflammation and fibrosis [160]. Pharmacological activation of PPARα increases CYP4A-driven ω-oxidation as well as peroxisomal and mitochondrial β-oxidation, leading to enhanced hepatic lipid turnover. Moreover, fibrates decrease the number of activated macrophages and stellate cells in the liver, and lower the expression of fibrotic markers [16].

The foz/foz (ALMS1 mutant) mouse model of Alström syndrome spontaneously exhibits a strong metabolic phenotype hallmarked by severe obesity, hyperinsulinemia and T2DM [161-163]. In this genetic background, PPARα activation reverses HFD-induced hepatocellular injury, liver inflammation and improves insulin sensitivity [164]. Similarly, Pparα-deficiency predisposes to HFD-induced increase in hepatic TGs, macrophage infiltration and elevated plasma levels of alanine aminotransferase (ALT) and SAA [165, 166].

The development of early stages of NASH was studied in the humanized APO-E2 knock-in (APO-E2KI) mouse. In this model, the Apo-E gene has been substituted for the human APOE2 allele under the control of the endogenous mouse promoter faithfully mimicking mouse endogenous APO-E tissue distribution and expression levels. The reduced affinity of hAPO-E2 for the LDL-receptor leads to a plasma lipoprotein profile similar to that occurring in human type III hyperlipoproteinemia (HLP) [159]. APO-E2-KI mice fed a western diet rapidly develop a phenotype characterized by steatosis and inflammation. Interestingly, macrophage infiltration in the liver precedes lipid accumulation. This is in contradiction with the concept that NASH pathogenesis stems from initial liver steatosis which leads to inflammation [167]. In accordance, clodronate liposome-induced depletion of residual liver macrophages (Kupffer cells), reduces hepatic TG content in HFD-fed wild-type mice [153]. Western diet-fed Pparα-deficient/APO-E2-KI mice manifest exacerbated liver steatosis and inflammation compared to wild-type APO-E2-KI mice, indicative of a protective role of PPARα against NASH [168]. Consistently, in primary hepatocytes isolated from APO-E2-KI mice, the HFD induces an

aberrant histone H3K9me3 and H3K4me3 methylation profile in the promoter of Pparα, which correlates with decreased Pparα mRNA expression [169]. In APO-E2-KI mice expressing PPARα, fibrates inhibit NASH due to their inhibitory effects on pro-inflammatory genes and the increase in lipid catabolism in the liver [167, 168].

Among the ROS, H_2O_2 is the major agent activating TGFβ and collagen production by hepatic stellate cells (HSC) [170, 171]. The anti-fibrotic action of synthetic PPARα agonists was demonstrated in a rat model of thioacetamide (TAA)-induced liver cirrhosis. PPARα directly upregulates catalyse expression thus ameliorating H2O2 detoxification and protecting hepatocytes from oxidative stress [172]. Moreover, fibrates improve endothelial dysfunction and ameliorate intrahepatic hemodynamics in carbon tetrachloride (CCl4) cirrhotic rats, at least in part, by reducing cyclooxygenase-1 (COX-1) protein expression [173]. In rodents, PPARα appears not expressed in endothelial and Kupffer cells, as well as in quiescent and activated HSC [174, 175]. Thus the anti-inflammatory and anti-fibrotic effects of fibrates in rodents depend on PPARα action within the liver parenchymal cells (Figure 2). Nevertheless, despite numerous reports of beneficial effects of fibrates in mice, species-specific differences exist in susceptibility to PPARα agonism. The relatively weaker efficiency of PPARα agonists in humans may be potentially due to a much lower expression level of PPARα in human liver compared to mouse liver [176, 177]. Several clinical studies however suggest beneficial effects of fibrates, hallmarked by decreased MRI-assessed steatosis and reduced levels of ALT/AST in patients with biopsy-confirmed NAFLD [178-180]. Another pilot trial showed no improvement in plasma enzyme parameters and histological NASH [181], thus larger studies evaluating fibrate action on a broad spectrum of liver pathologies, combining several methods of NAFLD assessment including liver histology, are still to be performed. Recently, GFT505, a novel dual PPARα/δ agonist [182] was shown to counteract multiple stages of

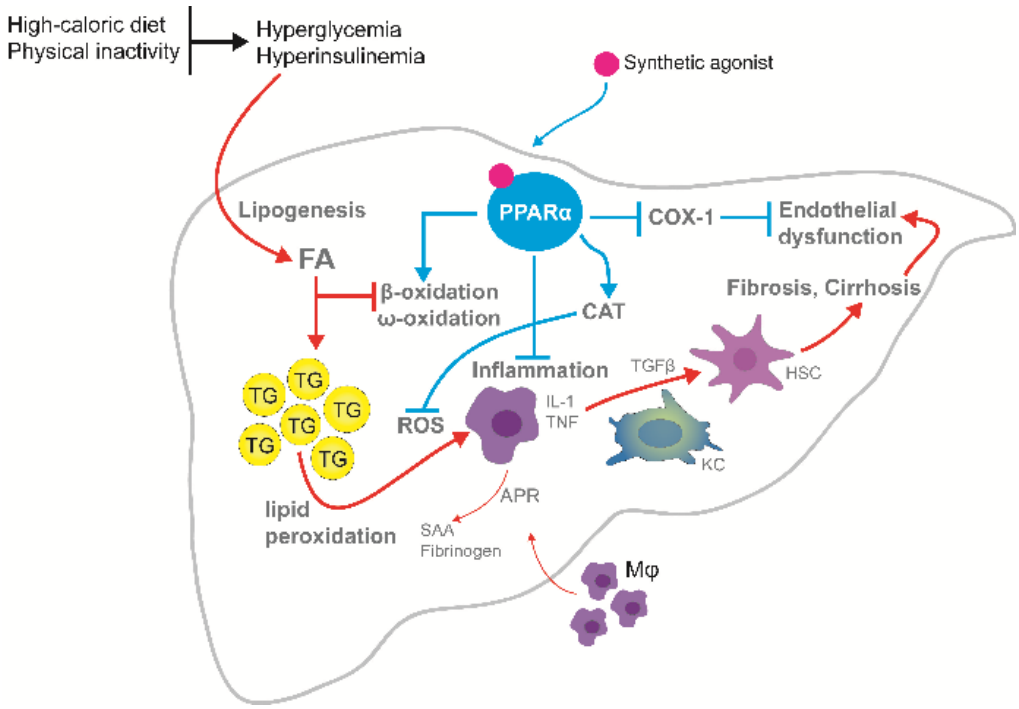

Figure 2. Hepatoprotective effects of fibrates: examples from rodent models of NAFLD. Development of NASH is provoked by different risk factors, such as western-type diet, physical inactivity and genetic predispositions that often lead to insulin resistanse and T2DM. Exaggerated food

intake leads to FA syntesis via hepatic lipogenesis pathways. Enhanced TG storage in liver (steatosis) provokes uncontrolled lipid peroxidation that generates reactive oxygen species (ROS) and cytotoxic aldehydes. Hepatocyte damage leads to increased inflammatory signaling (IL-1, TNF), acute phase response (APR) and recruitment of circulating (Mφ) and residual macrophages (KC). All of these mechanisms can directly induce apoptosis, necrosis and TGFβ-dependent activation of hepatic stellate cells (HSC) that are the main source of extracellular matrix protein in liver, thus contributing in fibrosis progression. In several mouse models of NAFLD, fibrate-activated PPARα counteracts different stages of NAFLD by promoting FAO and hampering pro-inflammatory response. Moreover, fibrate treatment induces catalase (CAT) expression thus diminishing H2O2 levels in the liver. Hepatic cirrhosis is associated with endothelial dysfunction and impaired intrahepatic hemodynamics that may lead to liver failure. Fibrates improve and ameliorate hepatic vascular resistance by reducing cyclooxygenase-1 (COX-1) protein expression.

NAFLD as assessed in several animal models of NASH and fibrosis [183]. GFT505 exerts preventive effects on liver steatosis and inflammation, induced in APO-E2-KI mice by a western-diet and in db/db mice by a MCD-diet. Furthermore, anti-fibrotic activities of GFT505 have been demonstrated on CCl4-induced fibrosis in rats [183]. In Phase II clinical trials, GFT505 treatment leads to decreased plasma concentrations of ALT, γGT and alkaline phosphatase (ALP) in MS patients [183]. Considering its ability to improve peripheral insulin sensitivity in abdominally obese patients as well as its TG lowering/HDL increasing activity in fasted subjects with combined dyslipidemia, GFT505 is a promising drug candidate for the treatment of diseases linked to insulin resistance, such as T2DM and NASH [182, 184]. Moreover, to improve the outcome of pharmacological therapy of NASH, dietary strategies, such as n-3 PUFA supplementation may be considered to ameliorate steatosis and inflammation, by a mechanism that partially relies on PPARα activation [18, 185-187].

CONCLUSION

PPARα-mediated induction of distinct gene clusters occurs as an adaptive response to multiple nutritional signals. In the fed state, PPARα activates lipogenic pathways enhancing FA storage used during periods of starvation when hepatic PPARα triggers multiple pathways of FAO and ketogenesis to yield energy substrates for peripheral tissues. In clinical practice, fibrates are PPARα agonists used in the treatment of hyperlipidemia, which efficiently decrease plasma TG and increase plasma HDL levels. Moreover, PPARα activation may contribute to an improvement of steatosis, hepatitis and fibrosis in mouse models of NASH, thus preventing liver failure. The possible usefulness of fibrates and n-3 PUFAs, which act at least in part via PPARα activators, in the treatment of NASH in human subjects remains to be demonstrated. Since fibrates are relatively weak PPARα agonists, highly potent and selective PPARα agonists and/or PPARα agonists with tissue-specific activity may be useful [188]. Amongst them, K-877 manifests greater efficacy than fibrates in term of its TG-lowering activity and ability to raise plasma FGF21 levels in Ldlr-deficient mice fed a Western diet [189]. Consistently, in Phase II clinical trials K-877 treatment more efficiently corrects fasting plasma TG and HDL-c in individuals with atherogenic dyslipidemia, in comparison with patients treated with fenofibrate [189]. Thus, K-877 could be a novel treatment option to tackle the residual cardiovascular risk. Aleglitazar is a dual PPARα/γ agonist displaying also a stronger normolipidemic activity than fenofibrate and improving insulin-sensitivity due to its PPARγ activity in T2DM patients [190]. Finally, the dual PPARα/δ agonist GFT505 is currently tested in Phase IIb trial for the therapy of NASH in metabolic syndrome and type 2 diabetic patients.

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Chapter 4

APPROACHES USED IN THE THESIS

In this section we briefly introduce selected techniques allowing *in vitro* PPARα characterization in order to assess its interactions with multiple transcription co-regulators and to characterize its ability to repress pro-inflammatory gene transcriptional activity in reporter gene assays. Further, the adeno-associated virus (AAV)-mediated gene transfer *in vivo* is described as the model of liver-specific *Pparα* reconstitution in *Pparα*-deficient mice.

PROTEIN MICROARRAY

To obtain a comprehensive overview of PPARα-coregulator interactions we applied the Microarray Assay for Real-time Coregulator-Nuclear Receptor Interaction (MARCoNI) developed by PamGene International. This protein chip allows testing NR-coregulator interactions for 155 peptide coregulator motifs that are immobilised on the porous ceramic membrane. The reaction mix composed of crude cell lysate containing green fluorescent protein (GFP)-tagged NR, the ligand and anti-GFP-FITC antibody is applied on the array to allow its binding with the peptides (Figure 1 b). The workstation performs series of washes with the sample solution to discard unbound NR. Interaction NR-coregulator peptide motifs can be monitored without and in the presence of the ligand by the measurement of the signal emitted by fluorescently labeled anti-GFP antibodies (Figure 1 c).

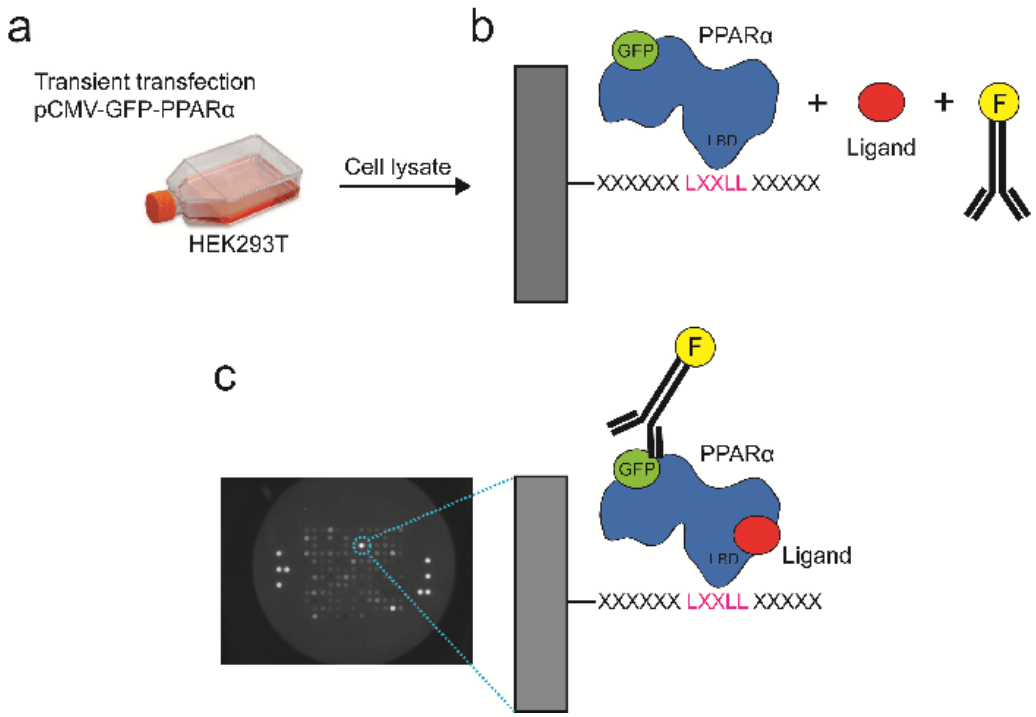


Figure 1. The workflow of PamGene protein chip. a. HEK293T cells were transfected with expression plasmid encoding GFP-PPARα. **b.** Cell lysate from transfected cells has been added to the reaction mix containing PPARα ligand and ant-GFP-FITC antibody and deposed on the protein array

to allow PPARα binding to immobilized peptide coregulator motifs. **c.** After series of washing the signal emitted by fluorescently labeled anti-GFP antibodies was digitalized and quantified using PS12 Pamgene machine.

To obtain recombinant PPARα, HEK293T (1 million cells, 80-90% confluence) were transfected with 5 µg of the plasmid encoding mouse PPARα tagged with GFP at the N terminus according to the manufacturer's protocol (jetPEI® transfection reagent, Polyplus transfection, NY, USA). 48 hours after transfection cells were rinsed with cold PBS and harvested in 200 µL of the lysis buffer (M-PER Mammalian Extraction Buffer, Thermo Scientific, IL, USA) containing 1:100 of Halt Phosphatase Inhibitor Cocktail and 1:100 of Halt Protease Inhibitor Cocktail EDTA free (Thermo Scientific, IL, USA) (Figure 1a). Samples were transferred to pre-cooled 2 mL tubes containing cell lysis stainless steel beads. Subsequently, samples were shaken twice during 1 minute 20 Hz using the Cell Disrupter Lyser. Crashed material was centrifuged 3 minutes at 1,000 g at 4°C. The supernatants were collected and centrifuged again for 45 minutes at 16,000 g at 4°C. Obtained supernatant was further used for the PamChip assay or snap-frozen liquid nitrogen and stored at -80°C. Protein quantification was performed with BCA Protein Assay Reagent (Thermo Scientific, IL, USA). 25 µg of total protein extract was applied per array.

Composition	Volume (µL)
ddH_2O	0.765
2x NR buffer (Pamgene)	12.5
DTT 0.05 mM	1.0
anti-GFP (FITC), Polyclonal (Thermo Scientific, PA1-46331), diluted 5 times	0.235
Ligand (Wy14,643 0.5 mM in DMSO)	0.5
Cell lysate (25 µg of protein)	10.0
Total per array	**25.0**

Table 1. The composition of protein chip master mix. The following protocol was run on PS12 Pamgene machine: NR_v05_50ms, NR_v05_100ms. The data analysis workflow, which includes image quantification, statistical analysis, visualisation and interpretation is performed with BioNavigator software.

TRANSREPRESSION ASSAY

The assess the ability of PPARα to repress transcriptional activity of pro-inflammatory genes such as IL-6 and fibrinogen-β *in vitro*, a firefly luciferase reporter gene assay was performed in the human hepatoma cell line (HepG2). Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 in DMEM culture media (4.5 g/L D-glucose) (Invitrogen, CA, USA) containing L-glutamine, sodium pyruvate, nonessential amino acids NEAA, penicillin-streptomycin and 10% FCS.

One day before transfection cells were split on 24-well plates, $5x10^3$/well. The next day, cells were transfected with 1 µg of DNA (Table 2) using 2 µL of jetPEI® transfection reagent per well, according to the manufacturer's protocol (Polyplus transfection, NY, USA). Transfection was performed in the presence of full-medium containing 10% FCS and antibiotics. Medium was changed 5 hours after transfection and cells were maintained for a further 19 hours. Cells were treated for 24 hours in the medium containing 0.2% FCS as indicated in Table 2.

a

vector	ng DNA	Treatment	Final concentration
pCAGGS	735	TPA (Phorbol 12-myristate 13-acetate)	200 nM
pGL3-AP-1-tk-luc	25	Wy14,643	10 µM
pRL-tk-Renilla	40		
pCAGGS-PPARα	200		
total	**1000**		

b

vector	ng DNA	Treatment	Final concentration
pCAGGS	110	Recombinant human IL-6	25 ng/mL
pGL3-hFibrinogenβ(-400)-luc	450	Wy14,643	10 µM
pRL-tk-Renilla	40		
pCAGGS-PPARα	400		
total	**1000**		

c

vector	ng DNA	Treatment	Final concentration
pCAGGS	360	Recombinant human IL-1	20 ng/mL
pGL3-hIL6-luc	200	Wy14,643	10 µM
pRL-tk-Renilla	40		
pCAGGS-PPARα	400		
total	**1000**		

d

vector	ng DNA	Treatment	Final concentration
pCAGGS	700	Wy14,643	10 µM
pGL3-NFκB-tk-luc	50		
pRL-tk-Renilla	40		
pCMV-p65	10		
pCAGGS-PPARα	200		
total	**1000**		

Table 2. The protocol of HepG2 transfection and treatment. a. cells were cotransfected with PPARα expression vector, pRL-tk-Renilla and pGL3 plasmid containing the AP-1 response element. Transcriptional activity of AP-1 was induced by treating cells with TPA (Phorbol 12-myristate 13-acetate). PPARα was activated by Wy14,643 **b.** cells were cotransfected with PPARα expression vector, pRL-tk-Renilla and pGL3-luc driven by the human fibrinogen β promoter (400 bp upstream of

the transcription start site). Transcriptional activity of fibrinogen β was induced by treating cells with recombinant human IL-6. PPARα was activated by Wy14,643 **c.** cells were cotransfected with PPARα expression vector, pRL-tk-Renilla and pGL3-luc driven by the human interleukin 6 (IL-6) promoter (1168 bp upstream of the transcription start site). Transcriptional activity of IL-6 was induced by treating cells with recombinant human IL-1. PPARα was activated by Wy14,643. **d.** cells were cotransfected with PPARα expression vector, pRL-tk-Renilla and a plasmid containing the NFκB response element. Transcriptional activity of NFκB was induced by cotransfecting cells with the plasmid encoding NFκB p65 subunit. PPARα was activated by Wy14,643.

Cells were harvested in the lysis buffer and the activities of firefly (Photinus pyralis) and Renilla (Renilla reniformis) luciferases were measured sequentially from a single sample using the Dual-Luciferase Reporter (DLR) Assay System (Promega, WI, USA) by the VICTOR Multilabel Plate Reader (Perkin Elmer, MA, USA).

AAV-MEDIATED GENE TRANSFER

Vectors based on the adeno-associated virus (AAV) are specific and non-pathogenic gene-delivery vehicles allowing long-term expression (up to 1 year or longer) of the transgene (>4.9 kb including both Inverted Terminal Repeat (ITR) sequences) *in vivo*. Different AAV serotypes reveal tissue-specific and cell-specific tropism (Table 3).

Serotype	Targeted tissue					
	Skeletal muscle	**Heart**	**Lung**	**CNS / Eye**	**Liver**	**Adipose**
AAV1	**X**	**X**	**X**	**X**	-	-
AAV2	-	-	-	**X**	-	-
AAV5	-	-	**X**	**X**	-	-
AAV6	**X**	**X**	**X**	-	**X**	**X**
AAV7	**X**	-	-	**X**	-	-
AAV8	**X**	-	-	**X**	**X**	**X**
AAV9	**X**	**X**	**X**	**X**	**X**	**X**
AAVrh10	-	-	**X**	-	-	-

Table 3. The tropism of selected AAV serotypes is shown in the following table with an X shown where efficacy has been demonstrated. Adopted from Penn Vector Core (http://www.med.upenn.edu/).

Evaluation of AAV vectors for liver-directed gene transfer in murine models revealed that the best performing vectors are AAV8. The transgene expression was driven by the hepatocyte-specific thyroxine binding globulin (TBG) promoter (Figure 2 a). The efficiency of AAV-gene delivery specifically in liver parenchymal cells was assessed in 8-week-old wild-type C57BL/6 males. AAV8-EGFP (Penn Vector Core, PA, USA) in sterile PBS solution (200 µL per mouse) was injected via tail vein in 3 concentrations (1E+11 GC, 3E+11 GC, 6E+11 GC) (Figure 2 b). Two weeks after injection mice were sacrificed, livers were removed, washed in PBS and fixed overnight in 4% paraformaldehyde PBS. Livers were then embedded in 20% sucrose overnight and frozen in OCT compound, in isopentane cooled with liquid nitrogen. For sectioning, the frozen tissue block was attached on the cryostat chuck and equilibrated to the cryostat temperature (-20°C) before cutting sections.Sections were cut at 7 µm and

picked up onto slides. Sections were dried at room temperature till the sections were firmly adherent to the slide and directly inspected under the microscope using normal FITC filter sets for viewing GFP (Figure 2 c).

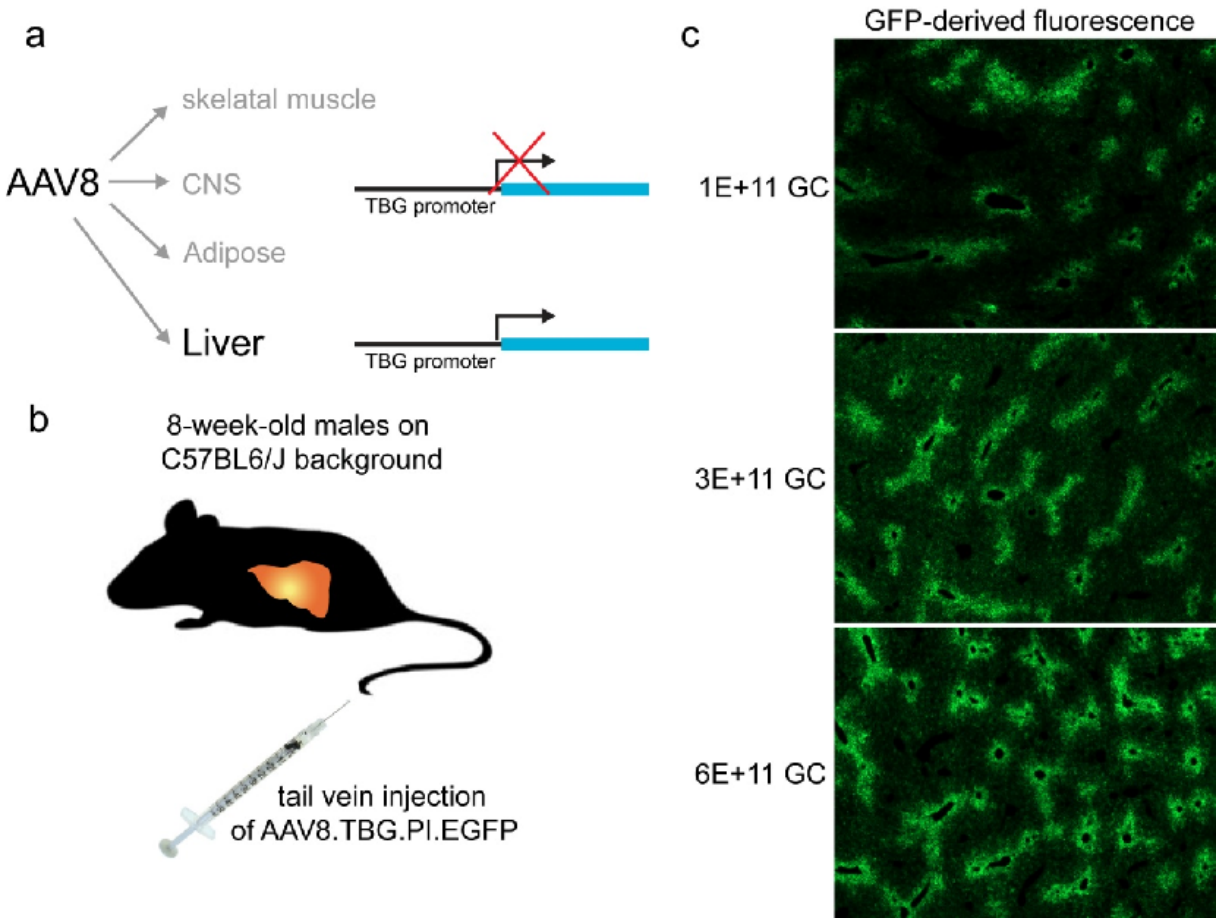


Figure 2. Liver-specific gene delivery *in vivo*. a. the thyroxine binding globulin (TBG) promoter allows hepatocyte-specific expression of the transgene. **b.** wild-type males were injected with AAV8-EGFP via the tail vein in order to test the specificity and efficiency of AAV8-mediated gene transfer. **c.** representative microphotographs of liver sections from mice injected with increasing doses of AAV8-EGFP.

In order to develop the model of liver-specific *Pparα* reconstitution in *Pparα*-deficient mice the coding DNA sequence (CDS) of mouse *Pparα* 5'-tagged with 3 FLAG sequence motifs (3XFLAG) was cloned into the pAAV2.1-TBG plasmid between the NheI and BamHI restriction sites (Figure 3).

The plasmid was maintained in recombinase minus (rec-) MAX Efficiency Stbl2 Competent Cells (Invitrogen, CA, USA) in the presence of carbenicillin and purified using an endotoxin-free method (e.g. Qiagen endo-free mega kit). The plasmid structure and integrity was validated by restriction enzyme analysis according to the requirements of Penn Vector Core (http://www.med.upenn.edu/). AAV8-PPARα vectors were produced by Penn Vector Core, PA, USA.

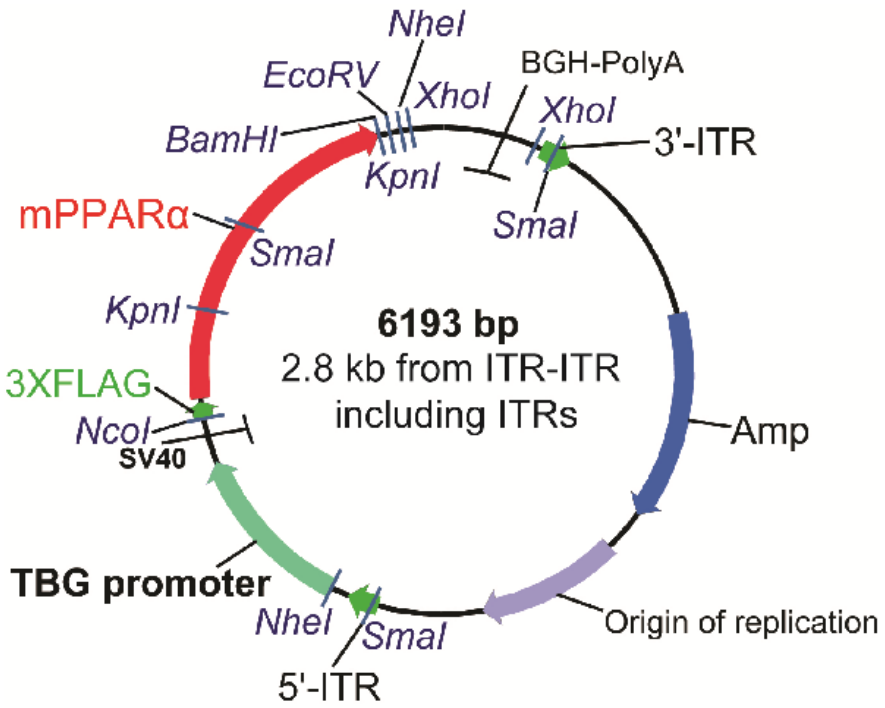


Figure 3. pAAV2.1-TBG-FLAG-mPPARα vector. The expression cassette of the plasmid comprises 2.8 kb including the TBG promoter, polyadenylation signal (polyA) and ITR sequences. Amp – ampicillin resistance gene. SV40 - Simian vacuolating virus 40 promoter.

Pparα-deficient 8-week-old males were injected with AAV8-PPARα and AAV8-EGFP in 3 concentrations (1E+11 GC, 2E+11 GC and 3E+11 GC). 2 weeks after injection the expression of PPARα was assessed by RT-qPCR SYBR Green technology (primers to specifically detect PPARα transcript in *Pparα*-deficient mice: forward 5'- CAG AGC AAC CAT CCA GAT GAC ACC-3'; reverse 5'- CGG ACT CGG TCT TCT TGA TGA CCT-3') and by Western blot with anti-FLAG M2-HRP monoclonal antibody (Sigma-Aldrich, MO, USA). A distribution of PPARα protein in the liver was assessed immunohistochemically by using anti-FLAG M2-FITC antibody (Sigma-Aldrich, MO, USA).

Chapter 5

PPARα INHIBITS PROGRESSION OF STEATOHEPATITIS TO FIBROSIS VIA A DNA BINDING-INDEPENDENT MECHANISM

ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is an increasingly prevalent liver condition characterized by excessive lipid deposition in the hepatocytes (steatosis) progressing to non-alcoholic steatohepatitis (NASH) is hallmarked by chronic inflammation. NASH markedly increases the risk of progression towards liver fibrosis, cirrhosis and hepatocellular carcinoma. The nuclear peroxisome proliferator-activated receptor alpha (PPARα) regulates hepatic fatty acid utilization and represses pro-inflammatory signaling pathways. Pharmacological activation of PPARα reverses diet-induced steatohepatitis, nevertheless, the relative contribution of lipid normalizing vs. anti-inflammatory activities of PPARα in NASH progression is unknown. Liver-specific expression of wild type or DNA binding-deficient PPARα in acute and chronic models of inflammation demonstrated that PPAR's anti-inflammatory, but not metabolic activities, result from DNA binding-independent mechanisms *in vivo*. We further show that PPARα inhibits the transition from steatosis toward NASH and fibrosis through a direct, anti-inflammatory mechanism independent of its effect on hepatic lipid metabolism.

Keywords: PPARα/PPRE/FAO/NASH/fibrosis

INTRODUCTION

Peroxisome proliferator-activated receptor alpha (PPARα/NR1C1) is a ligand-activated nuclear receptor controlling lipid utilization in tissues such as liver, heart and skeletal muscles. Through the transcriptional regulation of gene clusters controlling fatty acid oxidation (FAO) and energy utilization in the liver [1-3], PPARα maintains lipid and glucose homeostasis, and promotes ketone body synthesis upon food deprivation [4, 5]. PPARα also exerts anti-inflammatory effects during hepatic and systemic inflammation by repressing pro-inflammatory cytokine and acute phase gene expression through a tethering-based crosstalk with the AP-1, NFκB and C/EBPβ signaling pathways [6, 7]. The physiological importance of PPARα-driven transrepression has been demonstrated in cytokine-induced inflammation and the LPS model of endotoxemia [8, 9]. PPARα adopts the classical tertiary structure of nuclear receptors, with a C-terminal ligand binding domain (LBD) and a central, highly conserved DNA-binding domain (DBD) allowing the interaction of PPARα with specific DNA sequence elements termed Peroxisome Proliferator Response Elements (PPRE) [10]. PPARα forms chromatin-bound, transcriptionally activatable heterodimers with Retinoic X Receptors (RXRs) which are thought to play a major role in PPARα-mediated transactivation of its target genes [11]. While a variety of endogenous phospholipids and polyunsaturated fatty acids are believed to be endogenous PPARα ligands, fibrates are synthetic PPARα agonists used in clinical practice to lower plasma triglyceride levels and small dense LDL particles, and raise HDL-C levels in patients with dyslipidemia [12-14]. Interestingly recent studies indicate that PPARα agonism counteracts different stages of non-alcoholic fatty liver disease (NAFLD), a hepatic manifestation of the metabolic syndrome [15].

NAFLD which is hallmarked by chronic excessive triglyceride (TG) accumulation, affects 20-30% of the general population. Most patients remain asymptomatic, nevertheless some individuals may progress to non-alcoholic steatohepatitis (NASH), which is defined as steatosis accompanied by chronic hepatic inflammation and parenchymal cell ballooning [16, 17]. According to the multiple-hit model of NAFLD/NASH pathogenesis, lipid accumulation represents the first hit in NASH initiation, triggering oxidative stress, lipotoxicity and subsequent activation of hepatic inflammatory responses that further progress, in predisposed individuals, to more severe forms of liver pathology such as fibrosis, cirrhosis and hepatocarcinomas [18-22]. In murine models of dietary-induced NASH, fibrates reduce hepatic TG content and lipid peroxidation as well as reverse more severe NAFLD manifestations, such as steatohepatitis and fibrosis [23-25]. Accordingly, *Pparα* deficiency results in increased susceptibility to steatosis, oxidative stress and hepatic inflammation [26, 27]. Nevertheless, the relative contribution of the lipid normalizing vs. anti-inflammatory activities of PPARα in the control of NAFLD has not yet been studied. The hypothesis of this study was that metabolic actions of PPARα essentially result from PPRE-dependent gene activation, whereas anti-inflammatory actions are DNA-binding independent. To elucidate whether PPARα's ability to counteract hepatic NASH and fibrosis stems from its transactivating and/or transrepressing properties, we generated a PPARα R150Q mutant ($PPAR\alpha_{DISS}$) unable to bind PPRE motifs, but harbouring wild type-like interactions with co-regulator proteins and pro-inflammatory transcription factors. Accordingly, $PPAR\alpha_{DISS}$ is unable to trigger transcriptional activation of PPRE-driven genes in vitro and to increase the expression level of archetypical PPARα target genes in vivo, while it maintains transrepressing functions under pro-inflammatory conditions in vitro and in vivo. Liver-specific reconstitution of $PPAR\alpha_{DISS}$ in *Pparα*$^{-/-}$ mice treated with the PPARα agonist

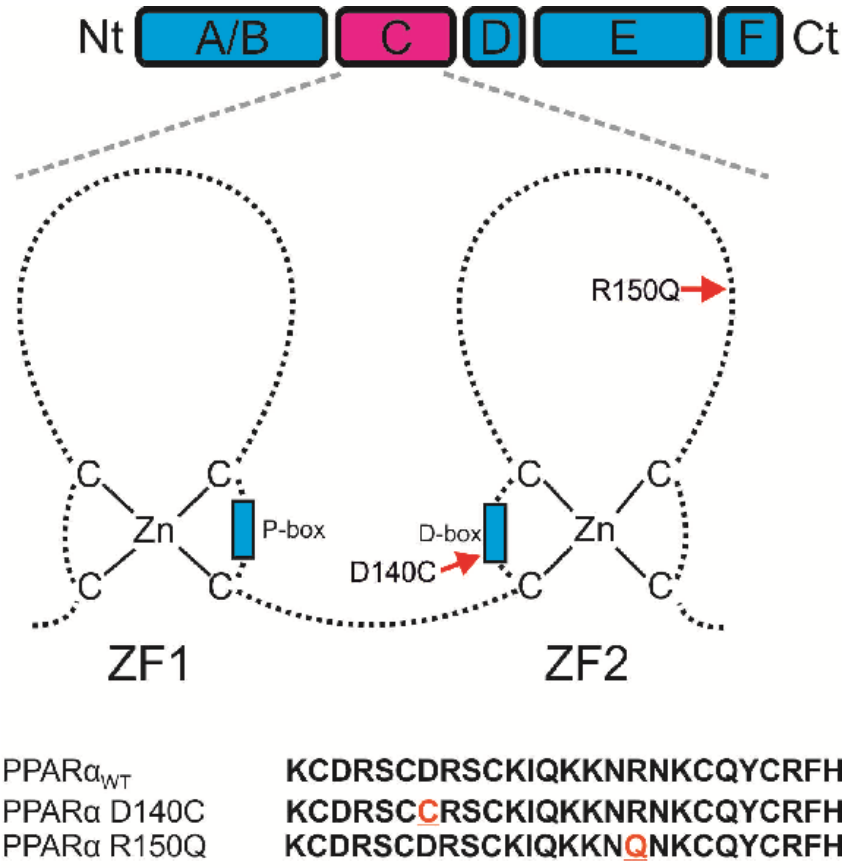
fenofibrate renders mice less responsive to the acute phase response induced by LPS. We further explored the properties of this DNA-binding crippled PPARα mutant in a model of chronic liver inflammation [methionine-choline deficient diet (MCDD)] which is histologically similar to human NASH [28]. In this dietary-induced steatohepatitis mouse model, hepatocyte-specific adeno-associated virus (AAV) reconstitution of PPARα expression showed that ligand-activated $PPAR\alpha_{DISS,}$ despite its inability to decrease intrahepatic lipid accumulation, protects against MCDD-induced liver damage and inflammatory response, to an extent comparable to that of $PPAR\alpha_{WT}$. Importantly, MCDD-fed *Ppar*$\alpha^{-/-}$ mice exhibited progressive pericellular hepatic fibrosis that was markedly reversed by pharmacologically activated $PPAR\alpha_{DISS}$.

The results thus show that PPARα inhibits the transition from simple liver steatosis toward a pathological state of NAFLD and fibrosis through a mechanism independent of its effect on hepatic lipid turnover, shedding new light on the role of PPARα in the multiple-hit model of NAFLD progression.

RESULTS

A targeted mutation within the DBD of PPARα abolishes its binding to PPREs

Analysis of the liver PPARα and RXRα cistromes [29] revealed that 92% of unique RXRα and 96% of shared PPARα/RXRα binding sites contain RXR or NR-RXR DR1 binding motifs, respectively. In contrast, only 61% of non-overlapping PPARα binding sites displayed classical NR-RXR motifs while showing significant enrichment in other binding motifs (Figure 1A) such as C/EBP or SP1, AP-1 and TCF12 which were less represented. In agreement with previous structural and functional studies, this analysis shows that RXRα-containing heterodimers, including PPARα-RXRα dimers, bind preferentially to DR1-like sequences. Importantly, it also suggests that PPARα might interact with genomic regions devoid of any PPRE-like sequences, pointing to potential tethering-mediated transcriptional regulatory mechanisms.



Supplemental Figure 1. Position and sequence of mutations introduced in the PPARα sequence. The two zinc fingers from the mouse PPARα sequence are depicted with its two main functional domains (P- and D-boxes). Positions of C140 and R150 are shown by red arrows. Sequences of wild type and mutated regions are shown (bottom panel).

To formally address this possibility, we generated PPARα mutants harboring a single amino acid substitution within the second zinc finger (ZF2) of the DBD, aiming at identifying a PPRE binding-deficient PPARα. The aspartate residue at position 140 was replaced by cysteine or arginine at position 150 by a glutamine residue, giving rise to the mutants PPARα$_{D140C}$ and PPARα$_{R150Q}$, respectively (Figure S1).

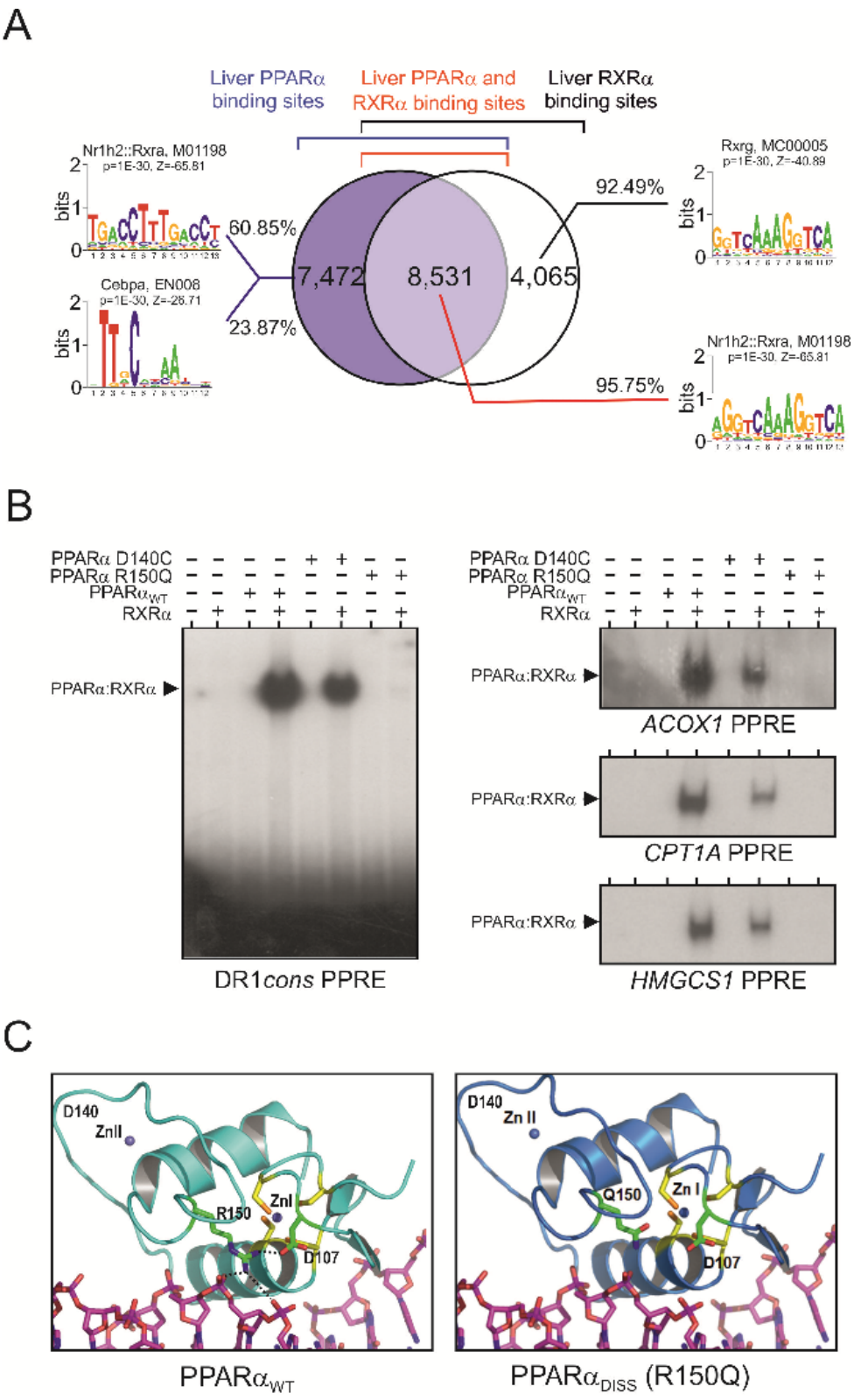


Figure 1. PPARα$_{DISS}$ mutant design and characterization. (A) Venn diagrams of PPARα and RXRα cistromes in mouse liver. Overlapping or unique genomic binding sites for PPARα and RXRα were searched for exclusive transcription factor binding motifs. Top overrepresented binding sites are indicated (% occurrence in total population). (B) DNA binding activity of PPARα mutants. The DNA binding activity of PPARα mutants was characterized by EMSA using various PPRE sequences. (C) The 3D structure of the PPARα was modelled and the impact of the R150Q substitution on the PPARα

structure shown. Dotted lines indicate non-specific interactions of R150 with the phosphate backbone of DNA and a salt bridge with D107. The loss of the positive charge upon R150Q substitution may destabilize PPARα-DNA interaction and the first zinc finger structure.

The DNA binding properties of these 2 ZF2 mutants were then investigated using electrophoretic mobility shift assays (EMSA) using various synthetic or natural PPRE-containing oligonucleotides corresponding to a DR1 consensus sequence and three natural PPREs from the acyl-CoA oxidase 1 (*ACOX1*), carnitine palmitoyltransferase 1 (*CPT-1)* and hydroxymethyl-glutaryl-CoA synthase (*HMG-CoAS*) gene promoters (Figure 1B, R. Mansouri, Thesis 2007). These assays revealed that PPARα$_{WT}$ and PPARα$_{D140C}$ were able to bind DNA in the presence of RXRα, whereas no binding could be detected for RXRα-PPARα$_{R150Q}$ dimers. PPARα$_{D140C}$ was thus dismissed from further studies, and PPARα$_{R150Q}$ was termed PPARα$_{DISS}$. Tri-dimensional structure simulations of the PPARα and PPARα$_{DISS}$ DBDs revealed discreet conformational changes within ZF2 of PPARα$_{DISS}$, leading to the loss of non-specific interactions between the DNA phosphate backbone and of a salt bridge with D107 (Figure 1C). This data indicate that substituting arginine at position 150 by glutamine, although inducing subtle conformational changes in the DBD structure, abolishes PPARα binding to PPREs.

PPARα$_{DISS}$ maintains its interactions with co-regulator proteins and heterodimerization partners

The acquisition of a transcriptional activity by PPARα requires both its dimerization with RXRα and the ligand-dependent recruitment of transcriptional coactivators to the PPARα LBD. Both properties mostly rely on the structural integrity of the C-terminal LBD [10]. We therefore compared the ability of PPARα$_{DISS}$ to that of PPARα$_{WT}$ to interact with RXRα and the canonical NR coactivator CBP to probe for the structural integrity of the PPARα$_{DISS}$ LBD. Both receptors exhibited a similar ability to dimerize, in a ligand-independent manner, with RXRα and to recruit CBP when liganded to the reference synthetic agonist Wy14,643 (Figure 2A-B). Of note, PPARα$_{WT}$ and PPARα$_{DISS}$ interacted similarly with the nuclear corepressor SMRT (data not shown). To investigate in-depth any potential allosteric transition in the LBD that could be triggered by the introduced R150 to Q mutation, a protein microarray approach was used to assess the ability of PPARα$_{DISS}$ to interact with a battery of co-regulator LXXLL motifs including those from CBP, SRC-1 and PGC-1α (Figure 2B). PPARα$_{DISS}$ physically interacted in a ligand-dependent manner with several LXXLL motifs from CBP, p300, MED1, SRC1, RIP140/NRIP1, PGC1α and TIF1α revealing a similar interaction profile as PPARα$_{WT}$. These interactions were not observed with PPARα deleted from its AF-2 domain (amino acids 450 to 468). The ligand-dependent transcriptional activity of PPARα$_{DISS}$ was investigated in reporter gene assays using different PPRE-driven promoter constructs (Figure 2D). As expected, co-transfection of the PPARα$_{WT}$ expression vector resulted in the induction of the luciferase reporter gene driven either by a consensus DR1 PPRE (DR1), or the *CPT-1α* and *ACOX1*-derived PPRE sequences. This effect was enhanced by co-transfection of RXRα and activation of PPARα$_{WT}$ by Wy14,643. Interestingly, PPARα$_{DISS}$ displayed neither basal nor ligand-induced transcriptional activity, irrespective of the promoter construct used.

Taken together, these results demonstrate that PPARα$_{DISS}$, despite preserving interactions with RXRα and multiple co-regulator motifs, is ineffective as a ligand-regulated transactivating factor.

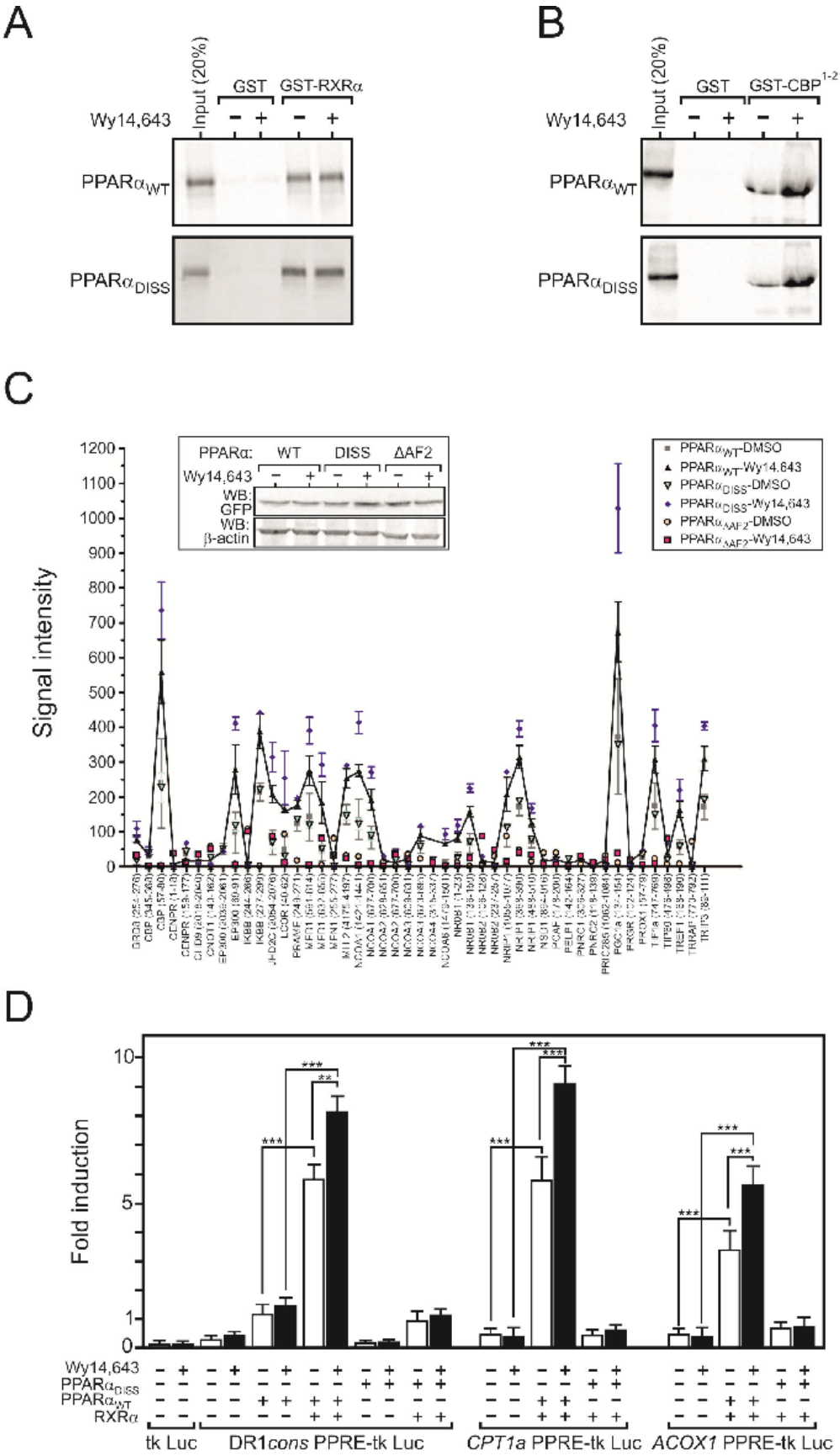
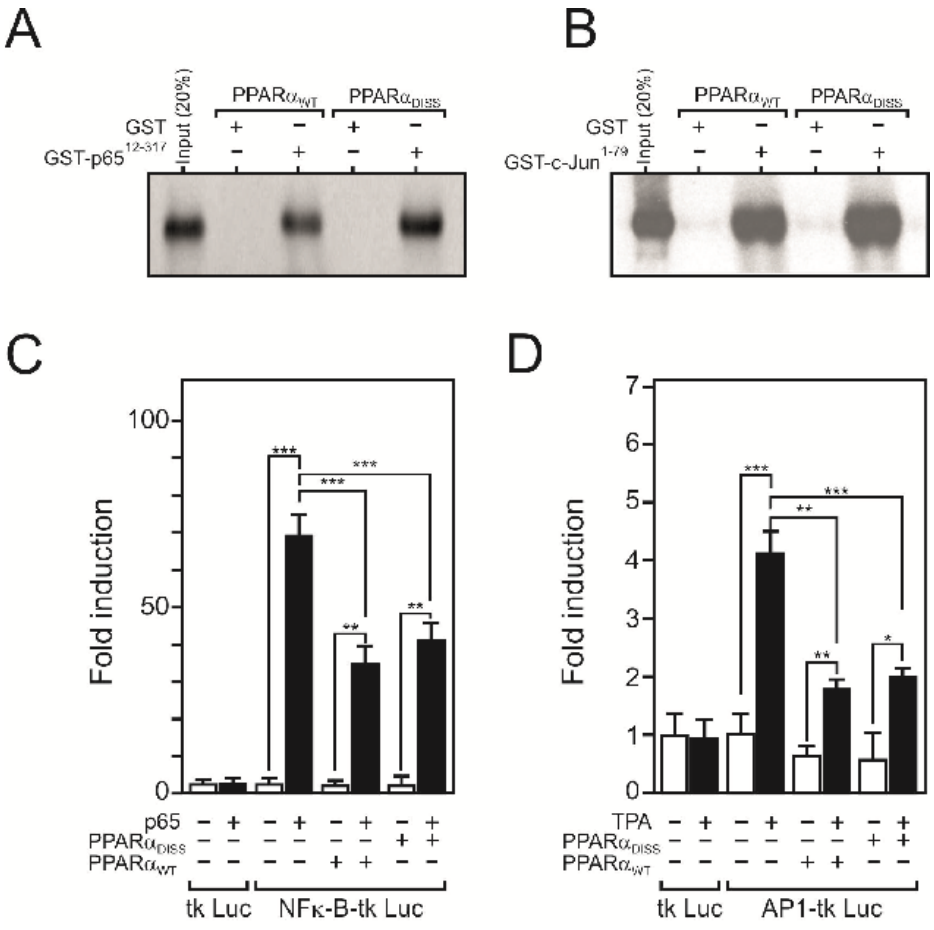


Figure 2. Structural integrity of the PPARα$_{DISS}$ mutant.

(A) Heterodimerization with RXRα. The interaction of PPARα$_{WT}$ and of PPARα$_{DISS}$ with its obligate heterodimerization partner RXRα was studied by GST-pull down assays. (B) Coactivator recruitment by PPARα$_{WT}$ and PPARα$_{DISS}$. The interaction of PPARα$_{WT}$ and of PPARα$_{DISS}$ with CBP was assayed in a GST-pull down assay. (C) Coactivator LXXLL motif interaction array. PAMChip arrays were used to interrogate PPARα$_{WT}$ and PPARα$_{DISS}$ interaction with a variety of LXXLL-containing motifs. The black line indicates interaction signals of PPARα$_{WT}$ with LXXLL peptides in the presence of Wy14,643. (D) Transactivation assays in a human hepatoma cell line. The activity of PPRE-driven reporter genes was monitored in HepG2 cells. tk Luc: negative control.

PPARα-driven repression of transcriptional activity of pro-inflammatory genes occurs via PPRE-independent modes.

Since the transcriptional interference of PPARα with the NFκB and the AP-1 pathways relies at least in part on PPARα's ability to interact with the p65 Rel homology domain and c-Jun [6], we examined by GST-pull-down assays whether the ability of PPARα$_{DISS}$ to interact with p65 (Figure 3A, R. Mansouri, Thesis, 2007) and c-Jun (Figure 3B, R. Mansouri, Thesis, 2007) was affected by the ZF2 mutation. PPARα$_{DISS}$ displayed wild type interaction with both transcription factors, suggesting that this mutant potentially retains its transrepressive capacity and could interfere with pro-inflammatory signaling pathways. This hypothesis was assessed in vitro using reporter gene assays in which the transcriptional activity of a NFκB- or an AP1-driven luciferase reporter gene was monitored in a human hepatoma cell line (HepG2, Figure 3C-3F). Co-transfection of PPARα$_{DISS}$ repressed the p65-mediated induction of the NFκB reporter gene, to an extent comparable to that of PPARα$_{WT}$.



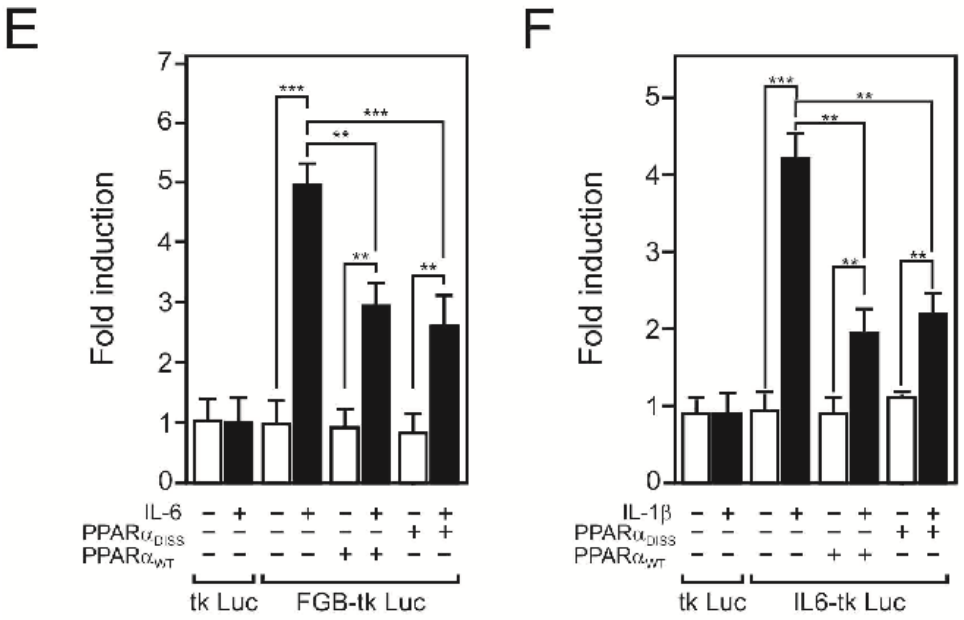


Figure 3. Transrepressive activity of PPARα$_{DISS}$. (A-B) PPARα$_{WT}$ and PPARα$_{DISS}$ interaction with the NFκB subunit p65 (A) or the AP1 subunit c-jun (B) was studied by GST-pull down assays. (C-D) The transcriptional interference of PPARα$_{WT}$ and PPARα$_{DISS}$ with the NFκB (C) or AP1 pathway (D) was evaluated by transient transfection assays in HepG2 cells (n=3-5). (E-F) Transcriptional interference with proinflammatory cytokine [IL-6, (E); IL-1β, (F)]-mediated activation of the *fibrinogen β* or of the *IL6* gene promoter respectively. **p<0.01, ***p<0.005, by ANOVA followed by a Tukey's post hoc test. Data are presented as mean ± SEM.

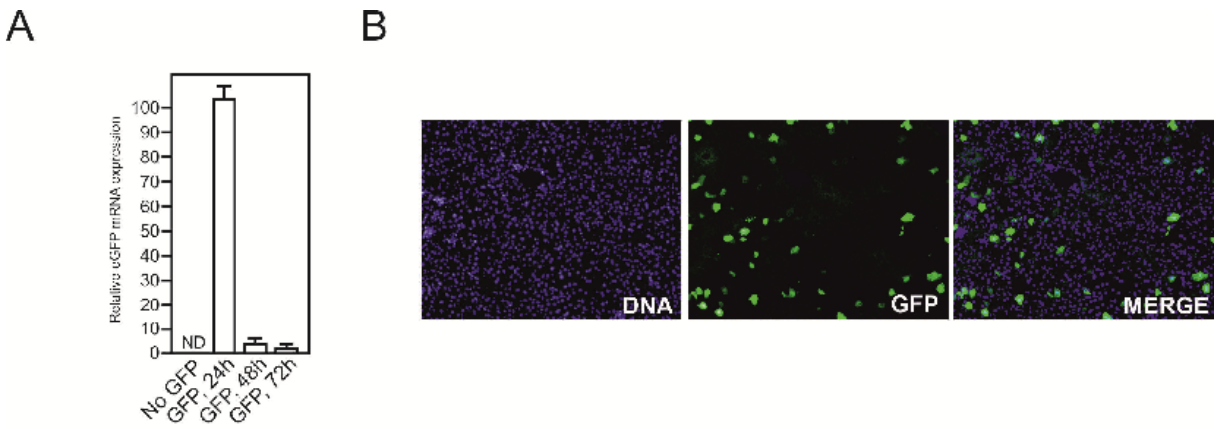
PPARα$_{DISS}$ also exhibited an unaltered transrepressive capacity on the TPA-induced transcriptional activity of an AP-1 response element-driven reporter gene, suggesting that PPARα$_{DISS}$ efficiently interferes with both the NFκB and AP-1 signaling pathways.

IL-1β and IL-6 are cytokines with profound roles in the acute phase response as well as in the development of chronic inflammatory responses in the liver [8, 9, 30]. To examine the potential impact of PPARα$_{DISS}$ on inflammatory cytokine-regulated signaling pathways, we studied its effect on IL-6-mediated transcription of human fibrinogen-β (*FGB*), an acute phase protein highly induced by IL-6 stimulation (Figure 3E). Induction of transcriptional activity of the *FGB* promoter reporter construct by IL-6 was prevented by co-transfection of either PPARα$_{WT}$ or PPARα$_{DISS}$. Furthermore, PPARα$_{DISS}$ abolished IL-1β-induced transcription activity of an IL-6 promoter-driven reporter gene (Figure 3F) showing that PPARα$_{DISS}$ is effective in transrepressing a representative range of pro-inflammatory gene promoters regulated through NFκB or AP-1 response elements. These findings suggest that subsets of the classical anti-inflammatory actions of PPARα are independent of its binding to PPREs, and are mediated at least in part by direct interaction with pro-inflammatory transcription factors.

PPARα$_{DISS}$ attenuates the acute phase response in LPS-induced endotoxemia

To investigate whether transactivating and transrepressing functions of PPARα may be dissociated in vivo, we evaluated PPARα's potency to behave as an active transrepressor in living animals using a LPS-induced endotoxemia model. PPARα$_{WT}$ or PPARα$_{DISS}$ expression

was reconstituted specifically in the liver of *Pparα*-deficient mice by hydrodynamic expression vector delivery as previously described [9]. The efficacy of hydrodynamic delivery was first assessed by delivering an expression vector encoding eGFP (Supplemental Figure 3).



Supplemental Figure 3. GFP expression in hydroporated mice. (A) Hepatic GFP gene expression levels in hydroporated mice 24, 48 or 72 hours after injection (n=3/group). (B) GFP protein expression in mouse liver 24 hours after hydroporation. DNA was stained with DAPI, and GFP localized by fluorescence.

As both hepatic mRNA (Supplemental Figure 3A) and eGFP protein expression (Supplemental Figure 3B) appeared most abundant 24-hours post plasmid injection, we designed a study to investigate the role of PPARα in hepatic acute inflammatory response (APR) under these conditions (Figure 4, E. Baugé, 2007). *Ppar*α-deficient mice were gavaged with fenofibrate (FF) and acute phase gene expression was induced by intraperitoneal injection of LPS, a potent initiator of APR and inflammation [31] (Figure 4A). In line with the in vitro transactivation assays, activation of PPARα$_{WT}$ by FF triggered the expression of *Acox1*, an archetypal PPARα target gene, whereas PPARα$_{DISS}$ was unable to trigger its induction (Figure 4A). Importantly, expression of PPARα$_{DISS}$ restored the inhibitory effect of FF on the LPS-induced APR gene expression to the same extent as PPARα$_{WT}$, as evidenced by the repression of the LPS-induced expression of 2 representative APR genes *serum amyloid A* (*Saa*) and *fibrinogen alpha* (*Fga*) (Figure 4C-D). Moreover, FF treatment prevented the hepatic, LPS-induced expression of the *Tnf*, pro-*Il-1β* and *Il-6* genes in mice expressing either PPARα$_{WT}$ or PPARα$_{DISS}$, in sharp contrast to empty plasmid hydroporated *Pparα*-deficient mice (Figure 4E-G). Interestingly, the observed decrease of hepatic cytokine gene expression was correlated with lowered plasma concentrations of the TNF, IL-1β and IL-6 cytokines (Figure 4H). These results thus support the view that PPARα hampers APR and inflammation in vivo through a mechanism that does not require direct binding to DNA via PPREs.

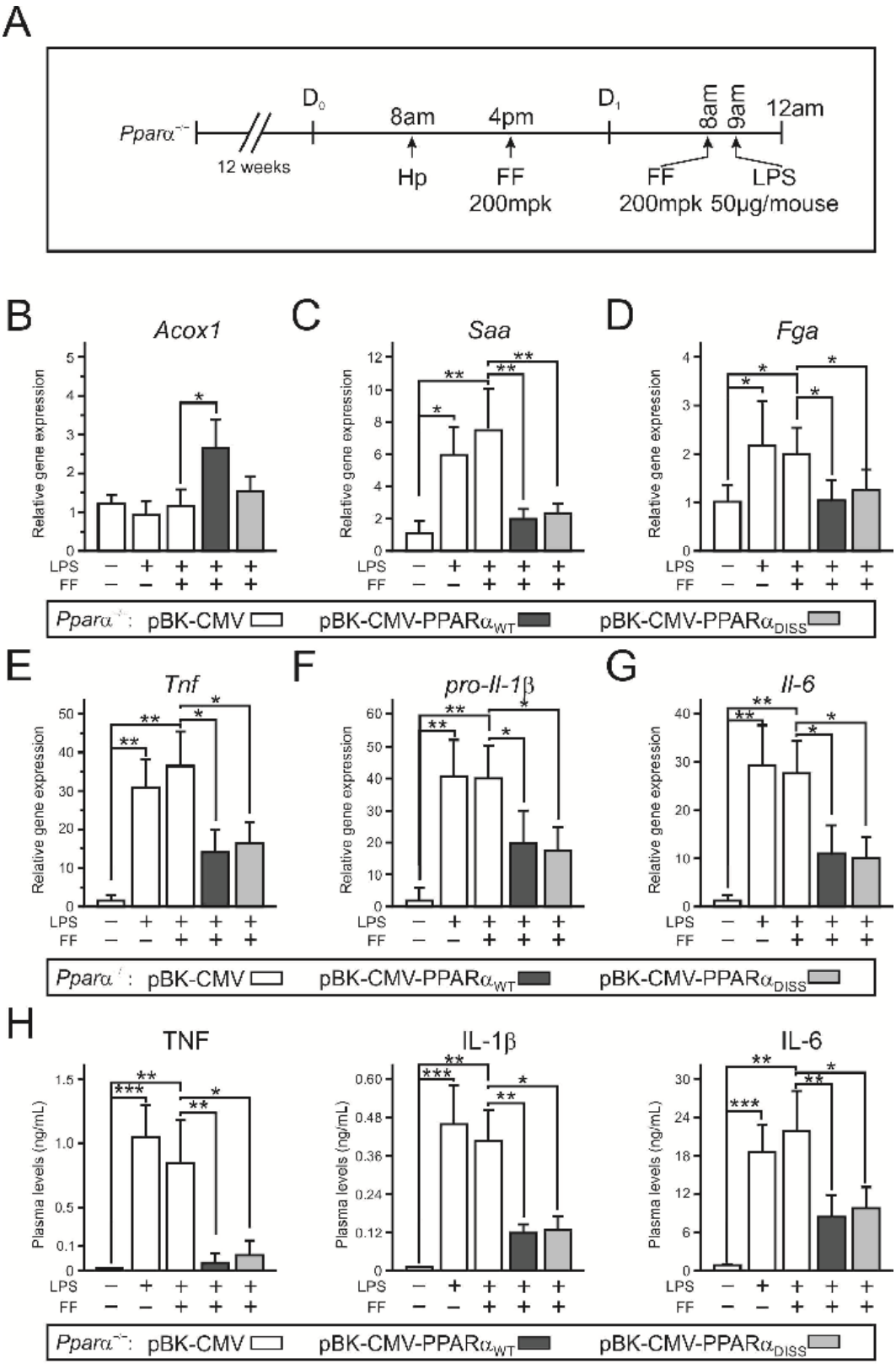
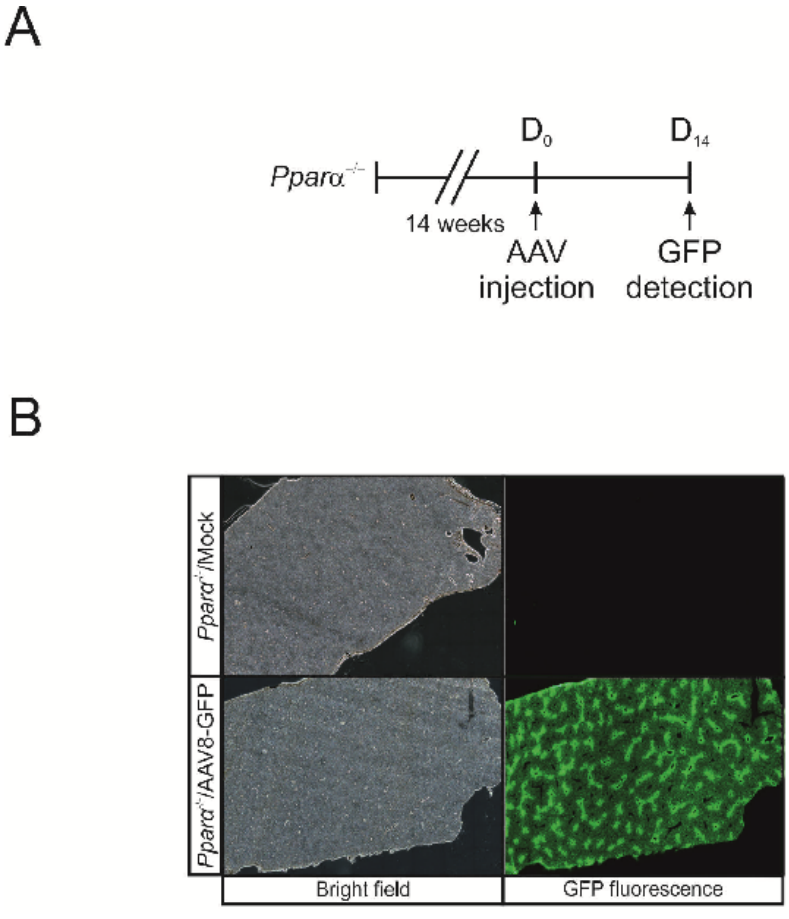


Figure 4. Anti-inflammatory activity of PPARα$_{DISS}$ in an acute model of inflammation. (A) Outline of the experimental procedure. (B-G) Hepatic gene expression in hydroporated mice treated or not with LPS and/or fenofibrate (FF). (H) Plasma levels of proinflammatory cytokines. Values are shown relative to mock-hydroporated, non treated mice (n=4-6/group). *p<0.05, **p<0.01, ***p<0.005, by ANOVA followed by a Tukey's post hoc test. Data are presented as mean ± SEM.

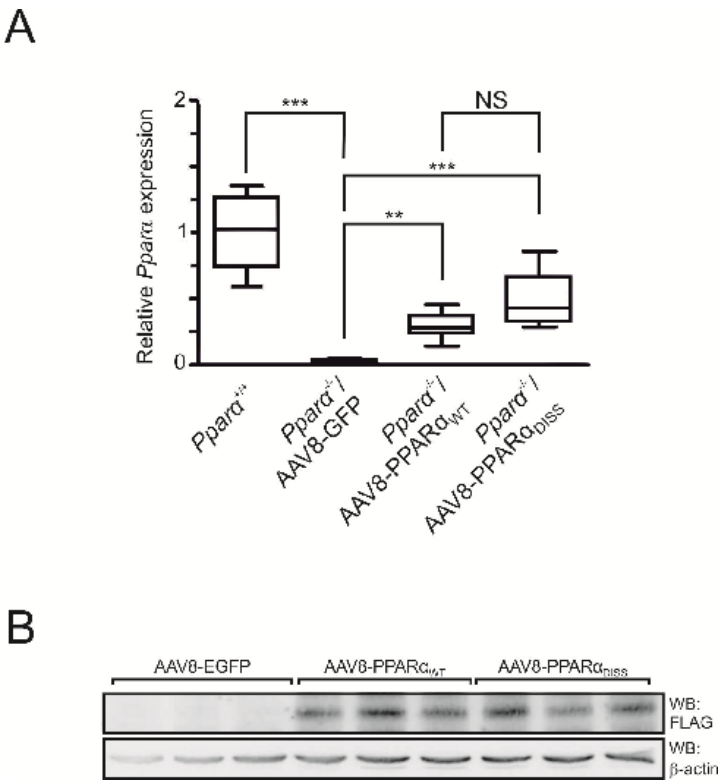
PPARα$_{DISS}$ protects against MCDD-induced liver injury and inflammation without affecting intrahepatic lipid turnover

Non-alcoholic steatohepatitis (NASH) is a chronic liver disease characterized by the presence of steatosis accompanied by chronic inflammation [20]. The mechanisms contributing to NASH progression remain elusive, nevertheless it was hypothesized that steatohepatitis results from reactive oxygen species (ROS) acting on accumulated intrahepatic fatty acids to yield pro-inflammatory lipoperoxides [23]. As PPARα activation by Wy14,643 has previously been found to effectively reverse nutritional-induced NASH in mice [23], we tested whether these effects stem from a PPARα-dependent transactivation and/or a transrepression mechanism. The chronic nature of this disease model prompted us to reconstitute hepatic PPARα expression in adult *Pparα*$^{-/-}$ mice using an adeno-associated serotype 8 virus (AAV8) expressing either PPARα$_{WT}$ or PPARα$_{DISS}$ driven by the thyroxine-binding globulin (*Tbg*) promoter (AAV8-PPARα$_{WT}$ or AAV8-PPARα$_{DISS}$, respectively). Such a strategy allows to specifically target liver parenchymal cells thus corresponding to PPARα expression pattern observed in wild-type mice [32, 33]. Liver distribution of the AAV8-delivered transgene was assessed histologically 2 weeks after injection of AAV8-eGFP, which proved to be efficient (Figure S4A, B).



Supplemental Figure 4. GFP expression after AAV8 injection to PPARα$^{-/-}$ mice. (A) Outline of the experimental procedure. (B) GFP protein expression in mouse liver 14 days after AAV8 tail vein injection. GFP was localized by fluorescence.

AAV8-PPARα$_{WT}$ or AAV8-PPARα$_{DISS}$ -injected mice were thus exposed for 23 days to a methionine-choline deficient diet (MCDD), which mimics the hepatic phenotype of human NASH [28]. The PPARα synthetic agonist Wy,14643 was coadministrated with MCDD for the last 5 days, whereas control animals received the same diet but containing methionine and choline throughout the experiment (Figure 5A). The efficacy of PPARα reconstitution in the liver of *Pparα*$^{-/-}$ mice was determined by assaying mRNA expression levels of PPARα, showing a ~30% restoration of PPARα$_{WT}$ or PPARα$_{DISS}$ expression relative to the expression of endogenous PPARα in wild type mice (Supplemental Figure 5A).



Supplemental Figure 5. Hepatic PPARα expression restoration after AAV8 injection in PPARα$^{-/-}$ mice. (A) PPARα expression was quantified by RT-QPCR in wild type mouse liver and compared to that assayed in liver from AAV8-GFP- (n=7), AAV8- PPARα$_{WT}$- (n=11) or AAV8-PPARα$_{DISS}$-injected (n=11) mice. Gene expression levels were measured 42 days after injection. Values are shown relative to wild type mice. Data are presented as box-and-whiskers plot showing median with 25th to 75th percentile intervals. (B) PPARα protein expression in liver of 3 mice/group 42 days after injection (including 28 days of MCDD treatment).

Western blot analysis of total liver extracts using an antibody against the FLAG-tag confirmed the presence of exogenous PPARα$_{WT}$ or PPARα$_{DISS}$ in AAV-injected mice (Supplemental Figure 5B). As previously shown [28], exposure to the MCDD led to weight loss and reduction of serum triglyceride and cholesterol concentrations (Supplemental Figure 6). Liver histology from MCDD-fed mice showed the ineffectiveness of Wy14,643 to counteract steatosis in *Pparα*$^{-/-}$ mice expressing hepatic AAV8-GFP, whereas activated PPARα$_{WT}$ protected mice from such an intrahepatic lipid accumulation (Figure 5B). In contrast, hepatic PPARα$_{DISS}$ was unable to counteract the MCDD-induced steatosis (Figure 5B). To examine whether histological changes in the liver of Wy-14,643 and MCDD-fed mice could be attributed to altered hepatic triglyceride (TG) handling, total hepatic TG levels were measured. AAV8-GFP and AAV8-PPARα$_{DISS}$ expressing mice showed a ~2-fold increase in

hepatic TG, whereas mice transduced with AAV8-PPARα$_{WT}$ were protected from TG accumulation (Figure 5C).

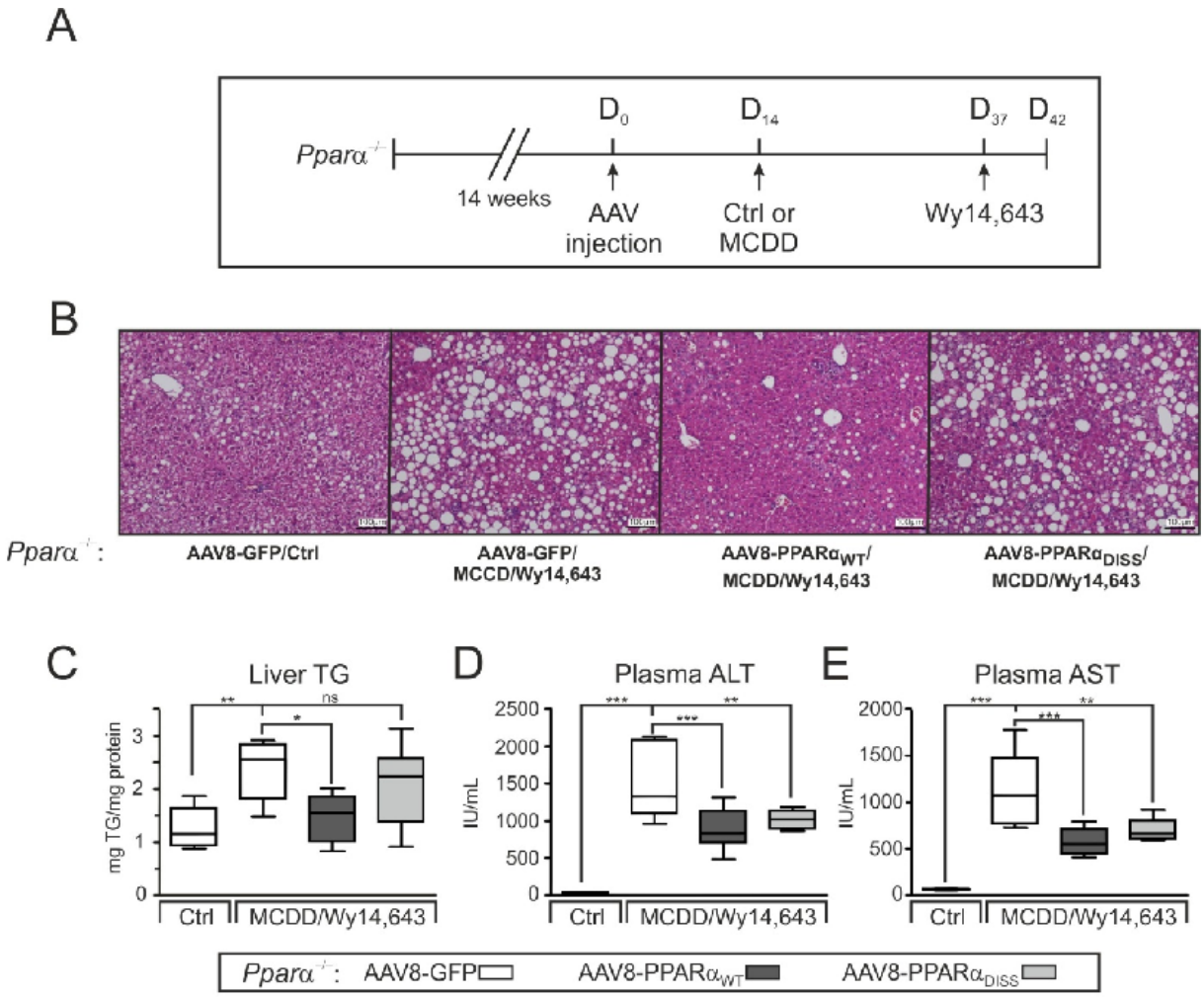


Figure 5. PPARα$_{DISS}$ does not prevent diet-induced hepatic steatosis. (A) Outline of the experimental procedure. MCDD: methionine and choline-deficient diet. Wy14,643 was administrated per os at 0.1% (w:w). (B) Hematoxylin and eosin (H&E) staining of liver sections after MCD diet feeding and WY14,643 treatment. (C) Hepatic triglyceride (TG) content and liver damage test assessment by measurement of (D) alanine aminotransferase (ALT) and (E) aspartate aminotransferase (AST) plasma levels. Values are shown relative to non-treated, AAV8-GFP-injected *Pparα*$^{-/-}$ mice. *Pparα*$^{-/-}$/AAV8-GFP (n=7/group), *Pparα*$^{-/-}$/AAV8-PPARα$_{WT}$ (n=11) or *Pparα*$^{-/-}$/AAV8-PPARα$_{DISS}$ (n=11). *p<0.05, **p<0.01, ***p<0.005, by ANOVA followed by a Tukey's post hoc test. Data are presented as box-and-whiskers plot showing median with 25th to 75th percentile intervals.

These results indicate that the preventive effect of PPARα agonism on liver steatosis and hepatic TG deposition is mediated by PPRE-dependent signaling pathways. Since elevated alanine aminotransaminase (ALT) and aspartate aminotransaminase (AST) levels reflect liver injury, we tested the effect of PPARα$_{WT}$ or PPARα$_{DISS}$ expression on serum ALT (Figure 5 D) and AST levels (Figure 5E). MCDD administration in *Pparα*$^{-/-}$/AAV8-GFP mice strongly elevated serum ALT and AST levels. Interestingly, both *Pparα*$^{-/-}$/AAV8-PPARα$_{WT}$ and *Pparα*$^{-/-}$/AAV8-PPARα$_{DISS}$ mice were protected against MCDD-induced liver injury as hallmarked by

significantly decreased ALT/AST serum levels (Figure 5D, E). Altogether, these data show that the hepatoprotective effects of activated PPARα are independent of its action on hepatic lipid turnover and can be controlled through a PPRE binding-crippled PPARα.

	Pparα$^{-/-}$			
Parameters	**AAV8-GFP**	**AAV8-GFP**	**AAV8-PPARα$_{WT}$**	**AAV8-PPARα$_{DISS}$**
Diet	***Control diet***	***MCDD/Wy14,643***	***MCDD/Wy14,643***	***MCDD/Wy14,643***
Number of animals	7	7	11	11
Weight (% change)	-0.18±1.62	-32.72±1.48***	-31.36±1.28***	-31.95±1.09***
Relative liver mass (% of body weight)	4.76±0.86	5.95±0.52	5.49±0.21	5.18±0.28
Free fatty acids (nmol/L)	0.49±0.07	0.51±0.04	0.67±0.05	0.84±0.07
Triglycerides (mg/dL)	106.5±9.3	36.1±5.5***	48.1±1.8***	35.0±1.5***
Total cholesterol (mg/dL)	162.3±15.5	33.4±3.0***	39.9±2.7***	34.2±1.1***

Supplemental Figure 6. Biometric and biochemical parameters of AAV8-injected mice. Data are presented as mean ± SEM. *Pparα*$^{-/-}$/AAV8-GFP (n=7/group), *Pparα*$^{-/-}$/AAV8-PPARα$_{WT}$ (n=11) or *Pparα*$^{-/-}$/AAV8-PPARα$_{DISS}$ (n=11). ***p<0.005, by ANOVA followed by a Tukey's post hoc test.

The hepatoprotective effects of PPARα$_{DISS}$ are mediated by a selective action on pro-inflammatory signaling pathways

The reduction of hepatic TG and prevention of steatosis has been linked to the ability of PPARα agonists to increase gene expression of fatty acid oxidation pathways [23]. Hepatic expression of peroxisomal acyl-coenzyme A oxidase 1 (*Acox1*, Figure 6A), peroxisomal L-bifunctional enzyme (*Bien*, Figure 6B) and cytochrome P450a10 (*Cyp4a10*, Figure 6C) was significantly elevated in MCDD-fed, *Pparα*$^{-/-}$/AAV8-PPARα$_{WT}$ mice treated with Wy14,643. In contrast, the expression of these genes, controlling FAO, was unchanged in *Pparα*$^{-/-}$/AAV8-GFP and in *Pparα*$^{-/-}$/AAV8-PPARα$_{DISS}$ mice, indicating that the lipid normalizing effects of hepatic PPARα stem from PPRE-dependent transactivation.

Cytokines such as IL-1β, IL-6 and TNF are involved in the pathogenesis of NASH and the transformation from simple liver steatosis to steatohepatitis and further liver injury [30, 34, 35]. Since interference of PPARα$_{DISS}$ with inflammatory pathways has been suspected from in vitro assays and established in vivo using the LPS endotoxemia model, we sought to determine whether the hepatoprotective effects of PPARα$_{DISS}$ relate to its impact on pro-inflammatory gene signaling. Wy14,643 treatment inhibited the MCDD-induced expression levels of *pro-IL-1β*, *Il-6* and *Tnf* in both PPARα$_{WT}$- and PPARα$_{DISS}$ expressing mice (Figure 6D-F). Interestingly, the expression of other genes characterizing an inflammatory response such as the chemokine *Ccl5/Rantes* and macrophage markers *F4/80* and *Cd14* were similarly downregulated by PPARα$_{WT}$ and PPARα$_{DISS}$ (Figure 6G-I). These results further suggest that PPARα exerts hepatoprotective effects by direct interference with pro-inflammatory signaling pathways by a mechanism complementary to its ability to control fatty acid catabolism.

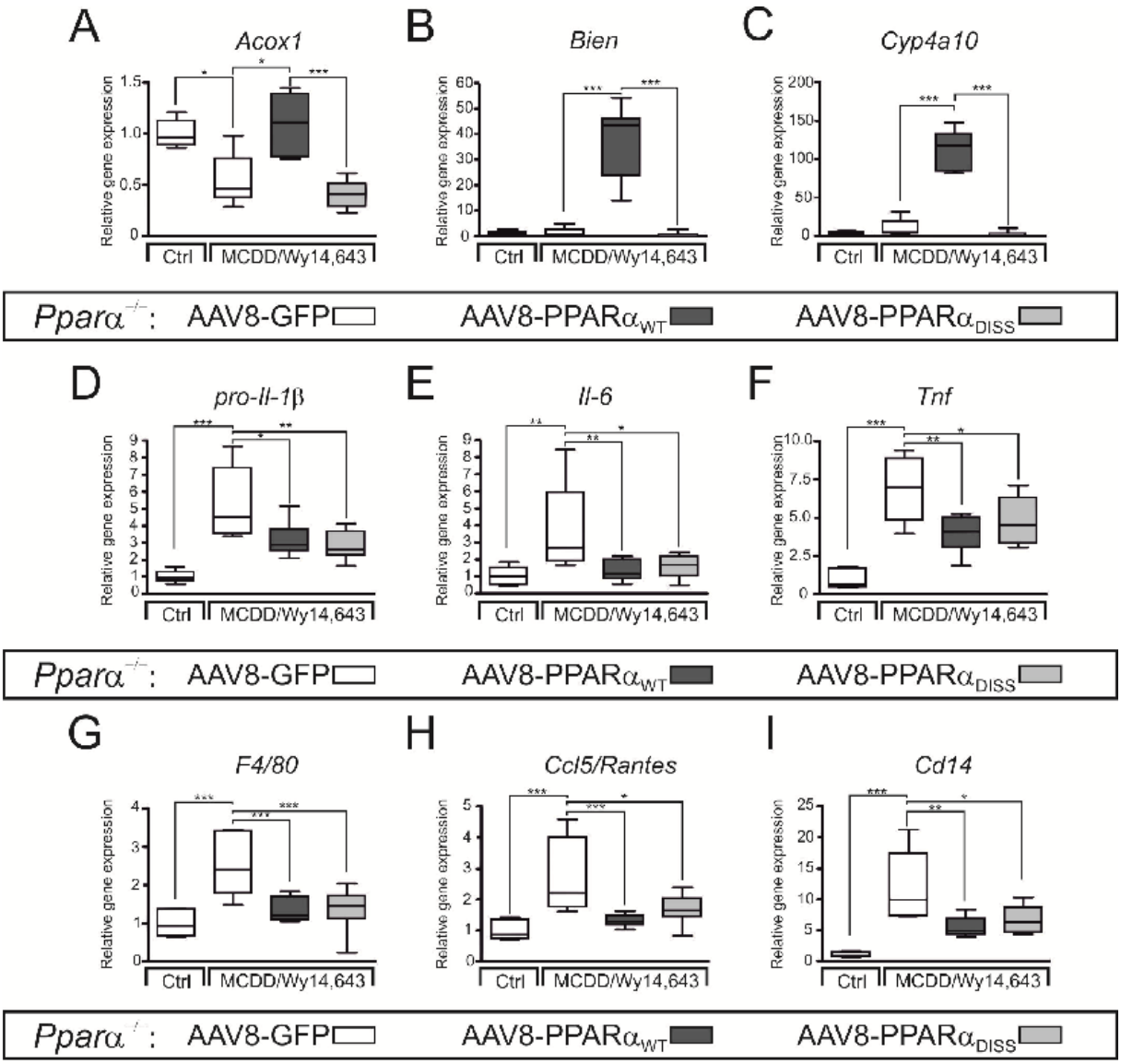


Figure 6. Hepatic gene expression of PPRE-driven metabolic genes and pro-inflammatory marker genes. (A-C) Hepatic expression of representative hepatic PPRE-driven fatty acid metabolism genes. (D-F) Hepatic expression of proinflammatory genes. (G-I) Hepatic expression of macrophage markers (*F4/80*, *Cd14*) and the chemokine *CCl5*. Values are shown relative to non-treated, AAV8-GFP-injected *Pparα*$^{-/-}$ mice. *Pparα*$^{-/-}$/AAV8-GFP (n=7/group), *Pparα*$^{-/-}$/AAV8-PPARα$_{WT}$ (n=11) or *Pparα*$^{-/-}$/AAV8-PPARα$_{DISS}$ (n=11). Data are presented as box-and-whiskers plot showing median with 25th to 75th percentile intervals. *p<0.05, **p<0.01, ***p<0.005, by ANOVA followed by a Tukey's post hoc test.

PPARα-mediated transrepression is sufficient to prevent liver fibrosis

Chronic liver diseases such as NASH predispose to the development of hepatic fibrosis, a clinical sign of liver injury [36]. We investigated whether ligand-activated PPARα$_{DISS}$ also influences hepatic fibrogenesis. As shown by Sirius Red staining of liver sections, MCDD feeding of *Pparα*$^{-/-}$/AAV8-GFP mice resulted in intraparenchymal pericellular fibrosis (Figure 7A). Expression of hepatic PPARα$_{WT}$ and PPARα$_{DISS}$ followed by Wy14,643 treatment in

MCDD mice led to a strong reduction in fibrosis as demonstrated by quantification of collagen staining (Figure 7B).

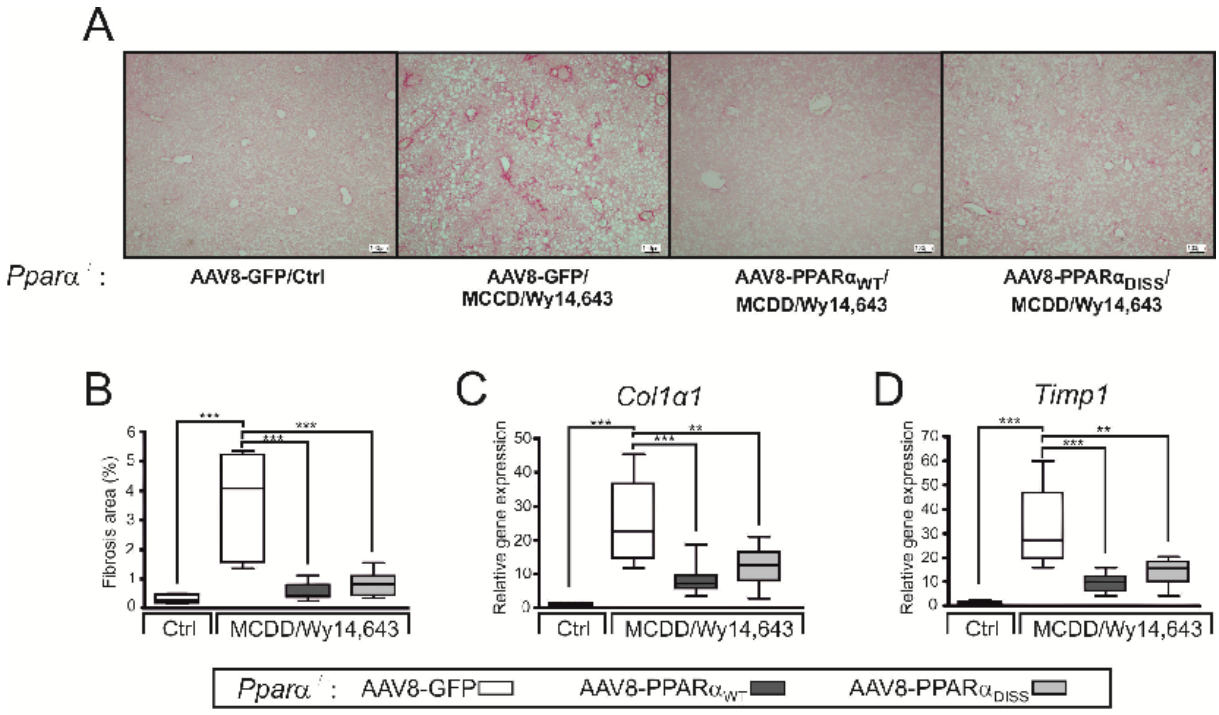


Figure 7. Liver fibrosis after hepatic PPARα restoration. (A) Hepatic collagen staining by Sirius Red. (B) Quantification of hepatic collagen deposition (n=8-11). (C-D) Hepatic gene expression of fibrosis markers. Values are shown relative to non-treated, AAV8-GFP-injected *Pparα*$^{-/-}$ mice. *Pparα*$^{-/-}$/AAV8-GFP (n=7/group), *Pparα*$^{-/-}$/AAV8-PPARα$_{WT}$ (n=11) or *Pparα*$^{-/-}$/AAV8-PPARα$_{DISS}$ (n=11). Data are presented as box-and-whiskers plot showing median with 25th to 75th percentile intervals. *p<0.05, **p<0.01, ***p<0.005, by ANOVA followed by a Tukey's post hoc test.

Thus both activated PPARα$_{WT}$ and PPARα$_{DISS}$ effectively preserve the liver from fibrosis development. In line with these results, MCDD feeding strongly induced the expression of genes involved in fibrogenesis such as collagen 1 α1 (*Col1α1*) and tissue inhibitor of metalloproteinase 1 (*Timp1*) in control *Pparα*$^{-/-}$/AAV8-GFP mice which was counteracted by Wy14,643 treatment in mice expressing hepatic PPARα$_{WT}$ or PPARα$_{DISS}$ (Figure 7C, D).

Taken as a whole, our data indicate that the anti-fibrotic activities of PPARα are mediated by a transrepressive mechanism which is unrelated to the control of hepatic fatty acid metabolism.

DISCUSSION

The results presented here provide evidence that modulation of the inflammatory response induced either by LPS, a TLR4 ligand, or by pro-inflammatory mediators during dietary-induced steatohepatitis can be efficiently counteracted through PPARα-mediated transrepression of signaling pathways controlled, amongst others, by AP-1 and NFκB transcription factors independently of PPARα binding to PPREs. Importantly, our results further demonstrate that NASH-induced fibrosis, an essential step towards even more severe clinical consequences such as cirrhosis and hepatocellular cancer, is prevented by PPARα agonism, independently of its effect on lipid accumulation in hepatocytes.

Nuclear receptor homo- or heterodimers establish contacts with two specifically arranged DNA half-sites via their respective DBD, a domain which is highly structured being composed by a pair of perpendicular α-helices stabilized by two C4 zinc finger domains [10]. Mutation (A458T) within the ZF2 of the mouse glucocorticoid receptor (GR) DBD yields a dimerization-defective GR ($GRdim^{(-)}$) harboring no direct binding to DNA yet potent anti-inflammatory activities, arguing for a tethered. transrepression mechanism of NFκB and AP1-regulated inflammatory pathways [37]. Whether such a mechanism also controls gene expression by other NR family members was yet unknown. Our findings demonstrate that $PPAR\alpha_{DISS}$ (R150Q) lacks DNA binding activity to consensus DR-1 and natural PPREs, while preserving interactions with RXRα and transcriptional coregulators. These data clearly establish that the loss of the transactivation potential of $PPAR\alpha_{DISS}$ both *in vitro* and *in vivo* is solely due to an alteration of the DBD structure. Furthermore, this mutant no longer regulates PPRE-driven target genes in vitro nor in vivo indicating that PPARα-controlled intrahepatic fatty acid catabolism requires interaction with PPRE sequences.

PPARα can also influence the transcriptional activity of several transcription factors via transrepression, a mechanism thought to be based on protein-protein interactions [6, 7]. Activation of the NF-κB transcription factor family, in concert with AP-1 dimers, plays a central role in inflammation through its ability to induce transcription of a range of pro-inflammatory genes including the cytokine genes IL1β, IL6 and TNF [37]. Here we demonstrate that $PPAR\alpha_{DISS}$ physically and functionally interacts with both the p65 and c-Jun components of the NF-κB and AP-1 transcription factors, respectively. Whereas being unable to induce expression of PPRE-driven target genes, PPARα behaves as a repressor of cytokine-induced AP-1- and NF-κB-driven transcription, demonstrating that PPARα remains a potent repressor of inflammatory signaling pathways *in vitro*. Our previous studies demonstrated the ability of PPARα to counteract the hepatic APR to IL1β and IL6 and LPS-induced inflammation [38]. LPS is the ligand inducing TLR4-mediated activation of a pro-inflammatory response, leading to IL1β and TNF release. We thus applied this model to investigate whether PPARα may exhibit dissociated transactivation/transrepression functions *in vivo*. Liver-specific restoration of both $PPAR\alpha_{WT}$ and $PPAR\alpha_{DISS}$ in a *Ppar*α-deficient genetic background was sufficient to attenuate LPS-mediated induction of inflammation and the APR, demonstrating that the control of pro-inflammatory signaling pathways by $PPAR\alpha_{DISS}$ stems from a transrepression mechanism. Therefore, our work validates the concept proposing that transactivation and transrepression mechanisms mediated by PPARα are dissociated entities. Of note, although PPREs could be found in the promoter of inflammatory response genes, it appears from our data that transrepression is responsible for the overall control of acute phase response of inflammation by PPARα [39].

Since the acute and chronic phase of inflammation are orchestrated by common mechanisms and mediators of cytokine signaling networks, it is reasonable to predict that these anti-inflammatory properties of PPARα are relevant for the modulation of chronic inflammation. Indeed, fibrates reversed dietary-induced steatohepatitis in pre-clinical studies [24]. This condition is a progressive chronic liver disease frequently associated with dyslipidemia, obesity and insulin resistance [23, 25, 27]. The exact mechanism contributing to the transition from simple liver steatosis to NASH, hallmarked by the presence of both steatosis and chronic inflammation, is not fully understood. However, it is hypothesized that hepatic fat accumulation triggers lipotoxic hepatocyte injury that further leads to induction of the inflammatory response [23, 26]. In line with this, recent studies suggested an important role of TLRs, which serve as pattern recognition receptors responsible for activation of immune cells in response to signals released upon tissue injury and death [40].

Prior studies documented a strong correlation between PPARα activity, intrahepatic lipid accumulation and development of more severe liver manifestations, such as steatohepatitis and fibrosis [24, 25]. Moreover, as previously shown and supported by this work, PPARα interferes with the AP1 and NFκB signaling pathways, which appear to be activated during the development of dietary steatohepatitis and liver fibrosis [41-43]. Nevertheless, the relative contribution of the lipid normalizing vs. anti-inflammatory activities of PPARα has not yet been studied in hepatic pathophysiology, prompting our investigation on the role of $PPAR\alpha_{DISS}$ in MCDD-induced NASH, which resembles the hepatic phenotype observed in humans [28]. As shown earlier and confirmed in our study, pharmacological activation of $PPAR\alpha_{WT}$ hampers MCDD-induced steatosis and hepatic TG levels, whereas Wy14,643 has no effect in mice lacking PPARα [23]. $PPAR\alpha_{DISS}$ showed no effect on intrahepatic lipid accumulation, an effect correlating with its inability to up-regulate expression of *Acox1*, *Bien* and *Cyp4a10* enzymes enhancing hepatic lipid turnover. In sharp contrast, $PPAR\alpha_{DISS}$ agonism efficiently prevented hepatocellular insult, as illustrated by decrease AST, ALT plasma levels, suggesting that PPARα prevents dietary steatohepatitis by a direct effect on inflammation rather than by stimulating hepatic fatty acid disposal as suggested in previous studies [23, 26].

Recent evidences showed that NAFLD progression to more severe forms such as steatohepatitis and liver fibrosis is mediated by inflammasome activity [43, 44]. Among them, NLRP3 is known to be activated by increased generation of ROS derived from enhanced lipid peroxidation [40]. NLRP3 activation leads further to caspase-1-dependent cleavage of effector pro-inflammatory cytokines, such as pro-IL-1β and pro-IL-18 [40]. IL-1β has, in turn, a prominent role in the progression from steatosis to steatohepatitis and liver fibrosis [30]. These observations are supported by the fact that mice lacking *Il-1β* are protected against liver inflammation [30]. Surprisingly, *Il1β*-deficient mice develop more pronounced liver steatosis indicating that inhibition of liver inflammation is not always correlated with reduced intrahepatic lipid content [30]. In the current model of MCDD-induced steatohepatitis, we show that PPARα devoid of its lipid normalizing activities markedly reduces liver gene expression of pro-inflammatory cytokines, including *pro-Il-1β*, *Il-6* and *Tnf* as well as other genes characterizing an inflammatory response, such as the chemokine *Ccl5/Rantes* and macrophage markers *F4/80* and *Cd14*. Recent studies show that activation of different inflammasome components during development of NASH may occur both in parenchymal and non-parenchymal cells [45] stimulating the production of transforming growth factor β (TGFβ) either by hepatocytes and Kupffer cells or by hepatic stellate cell (HSC) [46, 47].

TGFβ activates HSC functions, including the TGFβ autocrine loop, induction of actin reorganization and increase of collagen production, leading to development of liver fibrosis [44]. Although, as previously demonstrated, fibrates reverse dietary-induced fibrosis and hamper expression of several markers of HSC activation, they lack their activity to directly regulate TGFβ expression [24]. Therefore, it has been proposed that PPARα counteract fibrogenesis by limiting pro-fibrogenic stimuli deriving from lipid peroxidation [24]. Interestinlgy, our data show that ligand-activated $PPAR\alpha_{DISS}$ markedly reduces development of liver fibrosis as demonstrated by quantification of hepatic collagen and hamper pro-fibrotic gene expression including *Col1α1* and *Timp1* without an impact on intrahepatic lipid deposition. Thus anti-fibrotic effects of PPARα are driven by transrepression mechanism, either directly by repressing pro-fibrogenic gene expression or by diminishing upstream, pro-inflammatory signaling pathways.

Taken together, we demonstrate that PPARα inhibits hepatic inflammatory responses leading to liver injury and further development of fibrosis by a mechanism that relies on the transrepressive activity of PPARα, that appears to be independent of its binding to cognate PPREs. These findings highlight the potential of novel PPARα ligands in limiting the progression of chronic inflammatory liver diseases initiated by metabolic perturbations, through a direct counteraction of inflammatory responses independently of PPARα effect on intrahepatic lipid accumulation.

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EXPERIMENTAL PROCEDURES

Materials

Fenofibrate (FF) and Wy14,643 were purchased from Sigma-Aldrich (MO, USA). Mouse recombinant IL-1β and IL-6 were from PromoKine (Heidelberg, Germany). LPS (E. coli, serotype 055:B5) and phorbol 12-myristate 13-acetate (TPA) were purchased from Sigma-Aldrich (MO, USA).

Plasmids

PPARα (coding DNA sequence) CDS was inserted into pBK-CMV expression vector. pEGFP-C1-mPPARα expression vector [48] was a gift from Frank Gonzalez (N.I.H., Bethesda). The expression construct, pAAV2.1-TBG-3XFLAG-mPPARα containing the hepatocyte-specific thyroxine binding globulin (TBG) promoter was generated by inserting the 5'-tagged (3XFLAG) mouse PPARα cDNA with between the NheI and BamHI restriction sites. Single amino acid mutations were introduced in the PPARα CDS by site directed mutagenesis using the QuickChange II Kit (Stratagene, CA, USA). The aspartic residue at position 140 was replaced by cysteine ($PPAR\alpha_{D140C}$) or the arginine at position 150 was mutated to glutamine ($PPAR\alpha_{R150Q}$/$PPAR\alpha_{DIS}$). The firefly luciferase reporter vectors driven by PPREs composed of either the DR-1 consensus motif (AGGTCA A AGGTCA) or six copies of the native PPRE from the promoter from the human *APOA2* gene were as previously described [49]. Promoter-driven reporter genes were generated by cloning a 230 base pairs promoter fragment from the rat acyl-CoA oxidase (*ACOX1*) or a 600 base pairs genomic fragment of human carnitine palmitoyl-transferase I (*CPT-I*) into the pGL3 reporter plasmid (Promega, WI, USA). Fibrinogen-β promoter-, NFκB-response element-, AP-1 response element- and IL-6 promoter-driven reporter constructs were as previously described [6, 7, 50]. pRL-TK vector encoding the wild-type Renilla luciferase control reporter gene was purchased from Promega (WI, USA). The pRSV-p65 was previously described (9655393). pGEX vectors yielding full length RXRα, CBP^{1-213}, $p65^{12-317}$, $cJun^{1-79}$ and full length SMRT were described elsewhere [6, 51].

Mice, diets and AAV-mediated PPARα restoration

Experimental protocols were approved by the Lille Pasteur Institute ethical committee and carried out in agreement with European Union (EEC n°07430) and French ethical guidelines. For hydrodynamic gene delivery (hydroporation) 10-week old *Pparα*$^{-/-}$, weight-matched female C57Bl6/J mice fed a chow diet were rapidly injected via the tail vein with endotoxin-free plasmid DNA as previously described [9, 52]. Mice were gavaged twice with FF (200 mpk) in 1% carboxymethylcellulose (Sigma-Aldrich, MO, USA) 8 hours and 24 hours after hydroporation. One hour later, mice were subjected to peritoneal injection of saline LPS solution (50 µg per mouse). Animals were anaesthetized and sacrificed 3 hours post-LPS injection. Livers were removed and snap frozen in liquid nitrogen for mRNA analysis. Blood was collected on EDTA-coated tubes for plasma cytokine measurements.

AAV8-TBG-EGFP, AAV8-TBG-PPARα$_{WT}$ and AAV8-TBG-PPARα$_{DIS}$ vectors for persistent hepatocyte-targeted expression were produced by Penn Vector Core (PA, USA). 14-week-old, weight-matched male *Pparα*$^{-/-}$ mice were injected with 6E+11 genome copies of AAV8 per mouse. Two weeks after injection, the mice were fed a methionine-choline-deficient diet (MCDD) or a control diet [MCD supplemented with DL-methionine (3g/kg) and choline chloride (2g/kg)] for 28 days. *Pparα*$^{-/-}$/AAV8-GFP (n=7), *Pparα*$^{-/-}$/AAV8-PPARα$_{WT}$ (n=11) and *Pparα*$^{-/-}$/AAV8-PPARα$_{DISS}$ (n=11) received MCDD supplemented with Wy14,643 at 0.1% (wt/wt) during the last 5 days of the treatment as indicated and *Pparα*$^{-/-}$/AAV8-GFP (n=6) were fed a control diet. The mice were weighed and food was removed. Blood samples were taken after a 4 hour-fasting by retroorbital sinus punction under isoflurane anesthesia for free fatty acids (FFA), cholesterol, triglyceride (TG), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) measurements. Mice were sacrificed by cervical dislocation. Livers were weighed and the median lobes were fixed in 4% formaldehyde for histological analysis. The remaining lobes were snap-frozen in liquid nitrogen for mRNA analysis and TG quantification.

Chromatin immunoprecipitation (ChIP)-seq data analysis

Sequencing data files corresponding to PPARα (GSM864671) and to RXRα (GSM864674) genomic binding sites were downloaded as BED files from the NCBI Sequence Read Archive (SRA) and were described elsewhere [29]. All subsequent analysis were performed using the Galaxy Cistrome interface [53]. MACS algorithm was used at default value ($p<0.05$) for peak calling within the mouse genome (mm9). The "intersect" and "substract" algorithms were used to identify overlapping or unique PPARα and RXRα binding sites. The SeqPos motif tool was used to identify unique centered (-100bp/+100bp) motifs within each peak region.

Electrophoretic mobility shift assay

Mouse PPARα$_{WT}$, PPARα$_{D140C}$, PPARα$_{R150Q}$ and RXRα proteins were translated in vitro using the TNT T3 or T7 Coupled Reticulocyte Lysate System (Promega, WI, USA). Double-stranded oligonucleotides corresponding to the DR1-consensus, human hydroxymethyl-glutaryl-CoA synthase (HMG-CoAS), human *CPT-1* and *ACOX1* PPREs were used as indicated in the figure legends. In vitro translated proteins were pre-incubated for 10 min at room temperature in 20 µL of binding buffer (10 mM HEPES pH 7.8, 100 mM NaCl, 0.1mM EDTA, 0.5 mM DTT, 1 mg/mL bovine serum albumin, 10 % glycerol and 0.5 µg poly(dI-dC)). The radiolabeled probes (1 x 10^5 cpm of ^{32}P-end-labeled probe) were added to the binding reaction mix and incubated for 15 min at room temperature. Protein-DNA complexes were resolved on 6% non-denaturating polyacrylamide gels run in 0.25X Tris-Borate-EDTA, pH 8.0 at 150V for 2 hours at 4ºC and visualized by autoradiography.

Cell culture and reporter gene assays

HepG2 cells were grown in DMEM (4.5 g/L D-glucose) supplemented with 10% FBS, 2 mM glutamine, 100U/mL penicillin, 100 µg/mL streptomycin, 1 mM sodium pyruvate, and 1 mM non-essential amino acids. HEK293T cells were maintained in DMEM (4.5 g/L D-glucose) containing 100 U/mL penicillin and 100 µg/mL streptomycin in a humidified atmosphere at

5% CO_2 and 37°C. For reporter gene assays, HepG2 cells were transiently transfected with reporter and expression plasmids using the jetPEI transfection reagent (Polyplus-transfection, Illkirch, France) according to the manufacturer's instructions. After 24 hours of incubation, cells were treated with Wy14,643 (10 µM), IL-1 (10 ng/mL), IL-6 (20 ng/mL) and TPA (200 nM) as indicated in the figure legends. Luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, WI, USA). Transfections were carried out in triplicate, and experiments were repeated twice.

GST-pull down assays

GST and GST-fusion proteins were produced in BL21 E. coli (Life Technologies, Paisley, UK) and bound to glutathione-Sepharose 4B beads (GE Healthcare Life Sciences, Buckinghamshire, UK). Five µL of ^{35}S radio-labeled proteins synthesized using the TNT T3/T7 Coupled Reticulocyte Lysate System (Promega, WI, USA) were incubated with 0.5 µg of GST or GST-fusion protein in a total volume of 200 µL of incubation buffer in the presence of 100 µM Wy14,643 dissolved in DMSO or DMSO alone, as previously described [6]. After 2 hours of sample rotation at 4ºC, Sepharose beads were washed five times in incubation buffer supplemented with 300 mM NaCl, and separated by SDS-PAGE. After drying, the radioactive signal was recorded onto storage Phosphor-screen (GE Healthcare Life Sciences, Buckinghamshire, UK) and scanned on a STORM 860 PhosphorImager.

Co-activator array

The pEGFP-C1-mPPARα WT or pEGFP-C1-mPPARα$_{DISS}$ vectors were transfected into HEK293T cells using jetPEI transfection reagent (Polyplus-transfection, Illkirch, France) according to the manufacturer's instructions. 48 hours after transfection, cells were harvested in lysis buffer (M-PER Mammalian Extraction Buffer, Thermo Scientific, IL, USA) containing 1:100 of Halt Phosphatase Inhibitor Cocktail and 1:100 of Halt Protease Inhibitor Cocktail EDTA-free (Thermo Scientific). Samples were lyzed during 1 min at 20 Hz using the cell disrupter and centrifuged for 45 min at 16,000 g at 4°C. Protein quantification was performed on resulting supernatants with the BCA Protein Assay Reagent (Thermo Scientific, IL, USA). Twenty five µg of total protein extract was applied per array in the reaction buffer containing 2X NR buffer (PamGene Int., The Netherlands), 0.05 mM DTT, anti-GFP (FITC) goat polyclonal antibody (Thermo Scientific, IL, USA) and 10 µM Wy14,643 (in DMSO) or DMSO only as control. The co-activator array was run on the PS12 Pamgene machine and image quantification was performed with the BioNavigator software (PamGene Int., The Netherlands).

Messenger RNA analysis

Total liver RNA was isolated by Trizol Reagent (Life Technologies, Paisley, UK), treated with deoxyribonuclease I (Thermo Scientific, IL, USA) and transcribed into cDNA with the reverse transcription kit (Applied Biosystems, Warrington, UK). The real-time PCR measurement of individual cDNA was performed using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix

(Agilent Technologies, CA, USA) with the Mx3005 thermocycler (Agilent Technologies, CA, USA). Primer sequences are shown in Table S1. Expression levels were normalized to GAPDH gene expression as internal control.

Gene	Sense	Antisense	Accession number
Acox1	ACATCTTGGATGGTAGTCCG	TAACGCTGGCTTCGAGTGAG	NM_015729.2
Bien	TTGCCAATGCAAAGGCTCGT	GCAACAGGAACTCCAACGAC	NM_023737.3
Ccl-5	GTGCCCACGTCAAGGAGTAT	CCCACTTCTTCTCTGGGTTG	NM_013653.3
Cd-14	CCCGCAGTGAATTGTGACTA	CTGATCTCAGCCCTCTGTCC	NM_009841.3
Col1-α1	AGCACGTCTGGTTTGGAGAG	GCAGTGATAGGTGATGTTCTGG	NM_007742.3
Cyp4a-10	TGAGGGAGAGCTGGAAAAGA	CTGTTGGTGATCAGGGTGTG	NM_010011.3
F4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG	NM_010130.4
Gapdh	TGATGACATCAAGAAGGTGGTGAAG	TCCTTGGAGGCCATGTGGGCCAT	NM_008084.2
Il-6	CCAGTTGCCTTCTTGGGACTG	CAGGTCTGTTGGGAGTGGTATCC	NM_031168.1
Il-1β	GAATGACCTGTTCTTTGAAGTT	TTTTGTTGTTCATCTCGGAGCC	NM_008361.3
Pparα	CAGAGCAACCATCCAGATGACACC	CGGACTCGGTCTTCTTGATGACCT	NM_001113418.1
Timp-1	CCTTTGCATCTCTGGCATCT	CTCGTTGATTTCTGGGGAAC	NM_011593.2
Tnf	AGCACAGAAAGCATGATCCG	CCCGAAGTTCAGTAGACAGAAGAG	NM_013693.2

Table S1. Primer sequences for RT-PCR analyses.

Immunoblotting and ELISA

HEK293T and HepG2 cells or frozen livers were homogenized and sonicated in lysis buffer [50 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 1% nonidet-P40 and cOmplete Mini Protease Inhibitor (Roche, Basel, Switzerland)]. Total protein extract (50 µg) was separated by 8% SDS-PAGE and transferred onto nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK). Membranes were blocked using skim milk powder and then incubated overnight with specific primary antibodies against PPARα (sc-9000), GFP (sc-8334) or actin (sc-1616) as indicated. When indicated, a monoclonal anti-FLAG M2-HRP antibody (Sigma-Aldrich, MO, USA) was used and incubated during 1 hour at room temperature with the membranes. Peroxidase conjugated anti-rabbit immunoglobulin (Ig)G was used as secondary antibody for the detection of PPARα and GFP, and anti-goat IgG was used for actin. Immunolabeled proteins were visualized using the Femto Maximum Sensitivity Substrate (Thermo Scientific, IL, USA). Plasma levels of mouse IL-1 and IL-6 were determined by ELISA (R&D Systems, MN, USA) according to the manufacturer's instructions.

Plasma and liver parameters

Plasma free fatty acids (FFA), cholesterol, triglycerides (TG), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using a Konelab 20 Clinical Chemistry Analyzer (Thermo Fisher Scientific Inc., MA, USA). Liver TG content was determined as follows: frozen liver parts (50 mg) were homogenized in SET buffer (10 mM Tris-HCl, pH 7.4, 250 mM sucrose, 2 mM EDTA) and subjected to several freeze-thawing cycles. TG content was then assessed by enzymatic colorimetric method using TRIGL GPO/PAP reagent (Roche, Basel, Switzerland) and normalized to protein content determined by the BC Assay Kit (Interchim, France).

Histology

Liver histology was examined microscopically on paraffin-embedded sections (4 µM thickness) after hematoxylin and eosin (H&E) staining by standard procedures. Fibrosis assessment was carried out as follows: paraffin-embedded sections were stained with a 0.1% solution of sirius red in 1.3% saturated aqueous picric acid solution (Sigma-Aldrich, MO, USA). 15 microscopic fields of each liver section were randomly chosen and microscopically photographed at a 150-fold magnification. The area occupied by collagen was quantitated by morphometry using ImageJ [54] and expressed as a percentage of total cross-sectional area. To detect GFP distribution, livers were fixed in 4% formaldehyde, incubated overnight in 20% sucrose, frozen in OCT solution (Leica Biosystems, Nanterre, France) and viewed under fluorescence microscope after sectioning.

Statistical analysis

One-way ANOVA with Tukey's Multiple Comparison Test was used for all analyses. Values are presented as mean +/- SEM or Box-and-Whiskers plot showing median with 25^{th} to 75^{th} percentile intervals as indicated in the figure legends. Plots were created using the GraphPad Prism 5 software package (GraphPad Software, Inc. CA, USA).

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Chapter 6

LRH-1 PLAYS A CENTRAL ROLE IN HEPATIC TRIGLYCERIDE METABOLISM

Study performed in collaboration with Laboratory of Prof. Bert Groen, Departments of Pediatrics and Medicine of University Medical Center Groningen, The Netherlands.

ABSTRACT

The transcription factor liver receptor homolog-1 (LRH-1/NR5A2) has been shown to be of vital importance to sustain life in mice. In the liver, LRH-1 is important in the regulation of glucose and bile acid metabolism. Here we show that expression of the LRH-1 gene in liver biopsies of obese human subjects correlates negatively with the extent of NAFLD and NASH, indicating that LRH-1 also may play a crucial role in hepatic triglyceride metabolism. The causality of this effect was investigated in conditional whole-body *Lrh1* knockdown mice. We show here that *Lrh1* knockdown impairs PPARα signaling and decreases fatty acid β-oxidation and ketogenesis, whereas *in vitro Lrh1* overexpression induces *Pparα* expression. Conditional *Lrh1* knockdown mice develop a fatty liver phenotype, characterized by low circulating ketone bodies, high levels of plasma non-esterified fatty acids and hepatic steatosis in concordance with the human data. Conclusion: we show that LRH-1 plays a pivotal role in the control of hepatic triglyceride levels.

Keywords: Hepatic steatosis**,** Liver receptor homolog-1**,** Fatty acid β-oxidation**,** Peroxisome proliferator-activated receptor alpha**,** Non-alcoholic steatohepatitis

INTRODUCTION

Nutrient sensing transcription factors play a critical role in the maintenance of metabolic homeostasis in most species. The concerted action of the transcriptional network fine-tunes the interaction of an organism with its environment. The liver receptor homolog-1 (LRH-1/NR5A2) has a vital role in this network. Systemic disruption of the *Lrh1* gene in mice was shown to be embryonically lethal [1], but the vital importance of LRH-1 is not restricted to the embryonic phase. Recent data show that conditional *Lrh1* knock-out mice die 9-14 days after tamoxifen-induced disruption of the gene [2].

LRH-1 belongs to the NR5A family of nuclear receptors. It binds DNA as a monomer and is closely related to the orphan receptor SF-1. LRH-1 is expressed predominantly in liver and intestine, but also in pre-adipocytes, ovaria, pancreas and various other tissues [3, 4]. LRH-1 exerts diverse functions depending on its site of expression. In the liver, LRH-1 is involved in the regulation of bile acid and glucose metabolism [5-9].

Recently, it has been shown that LRH-1 is involved in the control of lipid metabolism as well [10]. Analysis of hepatic LRH-1 DNA binding sites by ChIP-seq followed by Gene ontology analysis revealed that LRH-1 binding occurs in proximity of genes related to lipid metabolism. Moreover, the presence of LRH-1 appeared to be required for the anti-steatotic effects of 1,2-dilauroyl-sn-glycero-3-phosphatidylcholine (DLPC; C12:0/C12:0)[11], a natural LRH-1 agonist[12]. These data suggest involvement of LRH-1 in the regulation of hepatic triglyceride metabolism. Abnormal accumulation of triglycerides in the liver, *i.e.*, hepatic steatosis, represents one of the hallmarks of the metabolic syndrome [13]. Hepatic steatosis or non-alcoholic fatty liver disease (NAFLD) can remain a benign, non-inflammatory condition without adverse effects, but may also progress to non-alcoholic steatohepatitis (NASH) and cirrhosis, conditions that severely compromise normal liver function and ultimately may result in end-stage liver disease and hepatic carcinoma [14].

To investigate the role of LRH-1 in the pathophysiology of human hepatosteatosis, we determined LRH-1 expression in liver tissue of a cohort of 125 extensively phenotyped obese patients with varying degrees of NAFLD and NASH. These data showed a negative correlation between LRH-1 expression and steatosis in human liver lipid metabolism. To further evaluate the role of LRH-1 in the control of hepatic lipid metabolism we used a conditional whole-body *Lrh1* knockdown mouse model [7]. The advantage of this model is that a hypomorph mouse of the *Lrh1* gene is generated that does survive for over a year. Moreover, due to the residual activity of LRH-1, regulatory network structure is kept intact as much as possible and less compensatory regulation of adjacent network genes can be expected. Our study shows that *Lrh1* knockdown (LRH-1-KD) disturbs hepatic triglyceride homeostasis primarily via downregulation of PPARα, resulting in decreased fatty acid oxidation and ketogenesis. LRH-1-KD mice develop hepatic steatosis specifically in periportal areas of the liver. Circulating ketone bodies are decreased, whereas plasma NEFA levels and medium- and long-chain acylcarnitines are increased. Thus, our data demonstrate that LRH-1 is an important regulator of hepatic lipid metabolism.

RESULTS

LRH-1 expression correlates with NASH in human liver

To study LRH-1 expression in human liver, LRH-1 mRNA levels were analyzed in liver biopsies of 125 patients presenting to the Antwerp University Hospital [15]. LRH-1 expression was significantly different between patients without or with the presence of NASH according to Brunt *et al.*[17] (p=0.016) with the lowest values in the NASH group. LRH-1 expression was also different according to the steatosis grade (p=0.011), the severity of the necroinflammation as reflected by the NASH Activity Score (NAS) (p=0.004) and fibrosis stage (p=0.014) with highly significant negative correlations in regression analysis (p=0.006, 0.001 and 0.023 for steatosis, NAS and fibrosis [18] respectively). Thus, LRH-1 expression negatively correlates with the severity of NAFLD and NASH in humans.

Phenotypic analysis of conditional LRH-1-KD mice

To further evaluate the role of LRH-1 in the control of hepatic lipid metabolism we used a conditional whole-body *Lrh1* knockdown mouse model. This model circumvents the early embryonic lethality caused by complete *Lrh1* deficiency, by using a conditional short hairpin RNA (shRNA) knockdown strategy [7]. *Lrh1* knockdown (LRH-1-KD) mice exhibited less than 5 percent of liver *Lrh1* expression compared to wildtype mice[7]. Such so-called hypomorphic alleles are very useful for studying essential genes at the organism level [19].

Chow-fed LRH-1-KD mice displayed a significantly increased liver weight compared to their wildtype littermates and hence an increased liver-to-bodyweight ratio (Table 1). Conversely, gonadal WAT mass was significantly reduced in the LRH-1-KD mice (Table 1).

Animal characteristics	Wildtype	LRH-1-KD	Change
Bodyweight (g)	28.7 (25.5 - 29.4)	24.8 (24.0 - 25.8)	
Liverweight (g)	1.0 (1.0 - 1.1)	1.3 (1.2 - 1.3)*	↑
Liver/bodyweight (%)	3.8 (3.7 - 3.9)	5.2 (4.7 - 5.4)*	↑
White adipose tissue weight (g)	0.8 (0.6 - 1.2)	0.3 (0.2 - 0.4)*	↓
White adipose tissue/bodyweight (%)	2.7 (2.5 - 3.5)	1.2 (0.8 - 1.2)*	↓
Plasma parameters	**Wildtype**	**LRH-1-KD**	**Change**
Triglycerides (mM)	0.10 (0.08 - 0.13)	0.12 (0.11 - 0.13)	
Total cholesterol (mM)	1.8 (1.7 - 1.8)	2.1 (2.0 - 2.3)	
NEFA (mM)	0.28 (0.20 - 0.35)	0.38 (0.36 - 0.44)*	↑
β-hydroxybutyrate (mM)	0.42 (0.42 - 0.59)	0.15 (0.14 - 0.27)*	↓
Hepatic metabolic parameters	**Wildtype**	**LRH-1-KD**	**Change**
Triglycerides (µmol/g liver)	19.0 (18.4 - 24.3)	34.0 (32.9 - 34.4)*	↑
Total cholesterol (µmol/g liver)	2.3 (2.1 - 2.6)	3.0 (2.9 - 3.6)*	↑
Free cholesterol (µmol/g liver)	1.9 (1.9 - 1.9)	2.7 (2.4 - 2.9)*	↑
Cholesterolesters (µmol/g liver)	0.5 (0.4 - 0.7)	0.6 (0.2 - 0.7)	
Phospholipids (µmol/g liver)	35.0 (34.0 - 50.3)	36.0 (35.5 - 40.0)	
Proteins (mg/g liver)	182 (146 - 223)	171 (164 - 173)	

Table. 1. Lrh1 knock-down results in liver TG accumulation. Different physilogical and plasma parameters of Wildtype and LRH-1-KD mice

Hepatic lipid content is increased by *Lrh1* knockdown

Hepatic total fatty acid and triglyceride levels were significantly elevated in LRH-1-KD mice compared to their wildtype littermates (Table 1, Figure 1A/B). No changes were observed in either hepatic protein, cholesterolester or phospholipid levels (Table 1): the latter implying that the increase in fatty acid content is solely due to triglyceride accumulation.

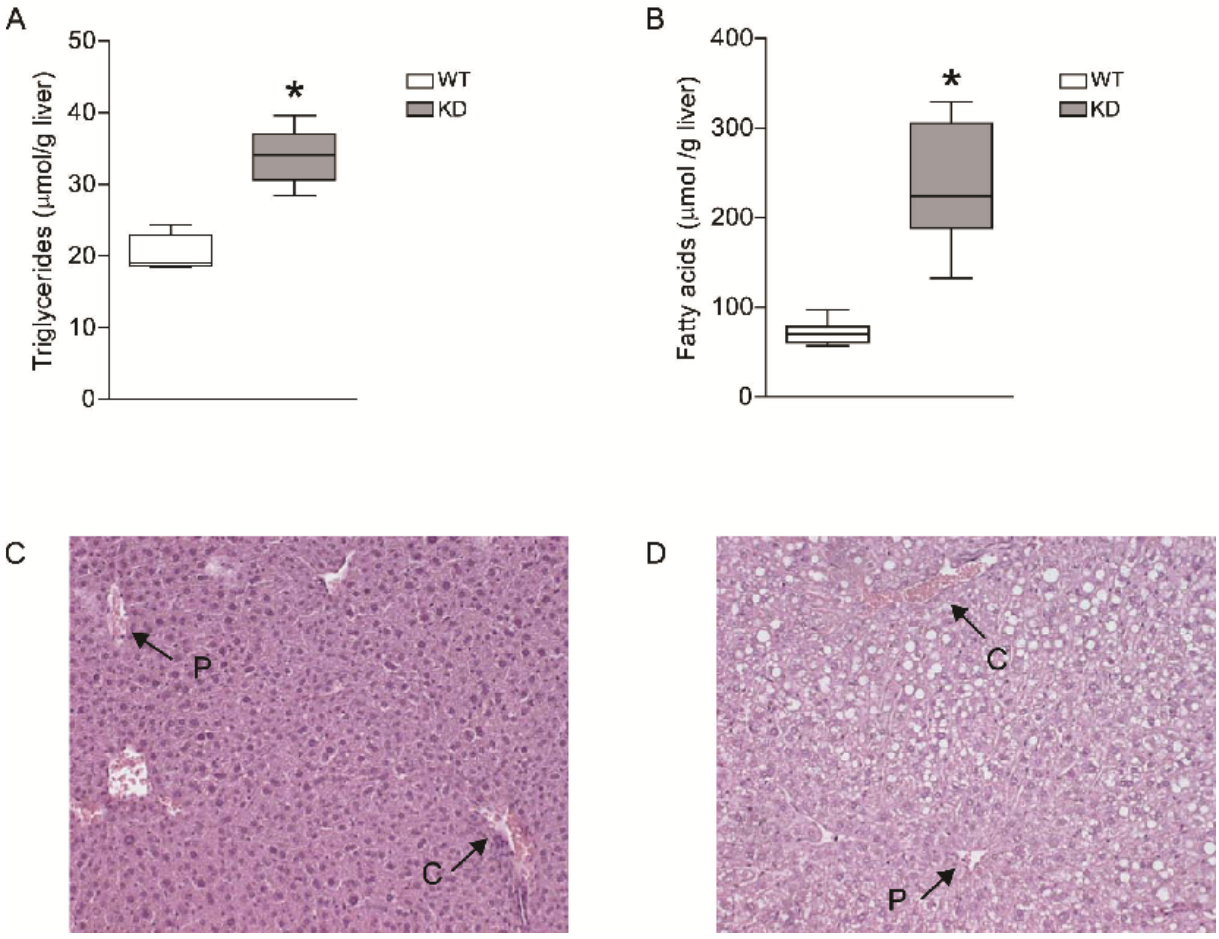


Figure 1. Lrh1 knock-down results in hepatic steatosis. (A) Hepatic triglyceride levels are increased in LRH-1-KD mice. (B) Hepatic fatty acid levels are increased in LRH-1-KD mice. Liver histology upon hematoxylin/eosin staining shows fat accumulation in livers of LRH-1-KD mice (D) but not in wildtype littermates (C), portal and central venes indicates as P and C respectively

Liver histology upon hematoxylin/eosin staining confirmed fat accumulation in livers of LRH-1-KD mice (Figure 1C/D). Fat appeared to be mainly deposited in periportal regions of the liver lobules, *i.e.*, the predominant site of oxidative energy metabolism [20]. Conversely, in perivenous zones, the predominant sites of *de novo* lipid synthesis [20], virtually no fat deposition was observed. As a control, no steatosis was observed in wildtype doxycycline-treated mice (Figure 1) or in mice expressing a control doxycycline-inducible shRNA against DCL1, a protein important for corticogenesis[21] (data not shown), indicating that doxycycline

treatment or the expression of a doxycycline-inducible shRNA did not cause the observed disturbance of hepatic triglyceride metabolism.

Lrh1 knockdown changes metabolic routes involved in lipid metabolism

In order to get insight in the mechanism underlying hepatic accumulation of triglycerides in LRH-1-KD-mice, a comparative Affymetrix microarray analysis was performed on liver tissue obtained from 4h fasted mice. Gene ontology analysis revealed that *Lrh1* knockdown affects transcriptional networks involved in inflammatory responses and lipid metabolism (Table 2).

Gene ontology

Function Annotation	p-value	# Molecules	% of gene set
lipid metabolic process	5,40E-14	63	10,3
defense response	1,90E-10	43	7,0
organic acid metabolic process	1,60E-09	43	7,0
cellular ketone metabolic process	3,10E-09	43	7,0
sterol metabolic process	1,30E-08	16	2,6
innate immune response	3,40E-08	18	2,9
regulation of response to stimulus	1,60E-07	31	5,1
response to other organism	4,20E-07	25	4,1
cellular lipid metabolic process	4,40E-07	38	6,2

Upregulated

Function Annotation	p-value	# Molecules	% of gene set
defense response	5,70E-14	37	9,6
response to other organism	6,40E-11	24	6,2
innate immune response	9,50E-11	17	4,4
regulation of immune system process	2,10E-10	27	7,0
positive regulation of response to stimulus	1,30E-09	20	5,2
positive regulation of immune response	3,60E-09	17	4,4
positive regulation of immune system process	7,30E-09	20	5,2
immune effector process	9,50E-09	16	4,2

Downregulated

Function Annotation	p-value	# Molecules	% of gene set
lipid metabolic process	5,70E-20	46	20,2
sterol metabolic process	9,00E-13	15	6,6
organic acid metabolic process	3,40E-12	30	13,2
cellular ketone metabolic process	5,90E-12	30	13,2
lipid biosynthetic process	7,80E-10	21	9,2
cellular lipid metabolic process	3,00E-09	26	11,4
lipid transport	2,40E-05	10	4,4
lipid localization	4,20E-05	10	4,4

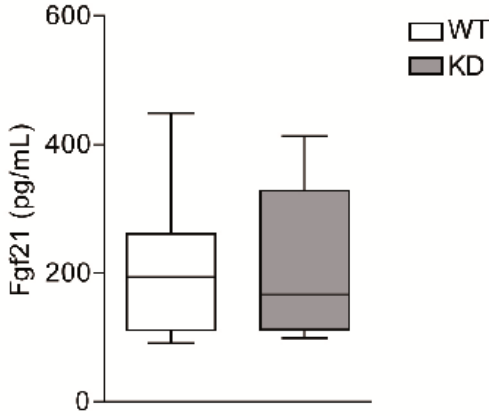
Lipid metabolic process

Gene ID	Name	Function
19013	peroxisome proliferator activated receptor alpha	nuclear receptor, transcription factor involved in lipid metabolism
56794	2-hydroxyacyl-CoA lyase 1	hydrolysis of fatty acids in peroxisomes
433256	acyl-CoA synthetase long-chain family member 5	lipid biosynthesis and fatty acid degradation
26897	acyl-CoA thioesterase 1	hydrolysis of long-chain acyl-CoAs of C12-C20-CoA in chain length to free fatty acid and CoA
320024	arylacetamide deacetylase-like 1	hydrolysis of lipids
12894	carnitine palmitoyltransferase 1a, liver	transport of fatty acids into mitochondria
76267	fatty acid desaturase 1 and 2	desaturation of fatty acids
20249	stearoyl-Coenzyme A desaturase 1	desaturation of fatty acids
22359	very low density lipoprotein receptor	metabolism of apoprotein-E-containing triacylglycerol-rich lipoproteins (such as VLDL)
56690	malonyl-CoA decarboxylase	malonyl-CoA to acetyl-CoA conversion

Table 2. Gene ontology analysis of *Lrh1* knockdown reveals transcriptional networks involved in inflammatory responses and lipid metabolism.

Several genes found to be upregulated upon *Lrh1* knockdown are mainly involved in inflammatory responses, which is consistent with previous studies showing that *Lrh1* has anti-inflammatory effects and negatively regulates the acute phase response [22-24].

Interestingly, downregulated genes appeared to be mainly associated with lipid and steroid metabolism. Careful analysis of this gene set revealed that many of the downregulated genes are actually involved in fatty acid catabolism (Table 2). Quantitative real-time PCR (qPCR) confirmed decreased expression of genes involved in fatty acid oxidation. Expression of *Pparα*, a lipid-sensing nuclear receptor and key regulator of fatty acid β-oxidation, ketogenesis and the adaptive response to fasting [25], was significantly decreased (Figure 2A). Also PPARα target genes involved in beta-oxidation and ketogenesis were downregulated, including carnitine palmitoyltransferase I (*Cpt-1a*), acyl-CoA oxidase (*Acox1*), HMG-CoA synthase (*Hmgcs2*) and fibroblast growth factor 21 (*Fgf21*) [26, 27] (Figure 2A), recently identified as a 'hepatokine' that is controlled by PPARα and stimulates hepatic fatty acid oxidation and ketogenesis [26-30]. However, plasma Fgf21 levels were not different between LRH-1-KD mice and wildtype mice (Figure S1).



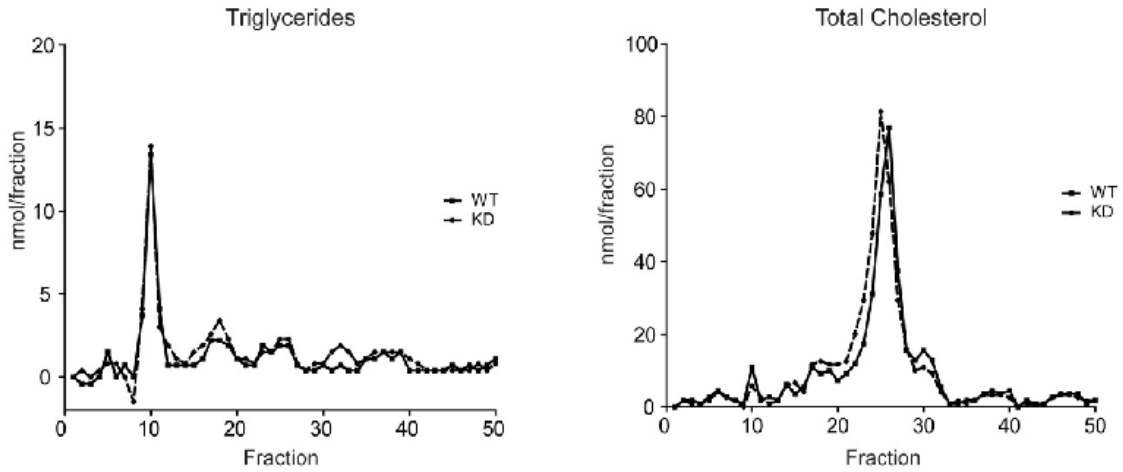
Supplemental figure 1. Plasma Fgf21 levels are unchanged. Fibroblast growth factor 21 levels in plasma of LRH-1-KD versus control mice.

Lrh1 knockdown decreases hepatic oxidation of fatty acids

Thus, knockdown of *Lrh1* appears to affect hepatic lipid metabolism, particularly the breakdown of fatty acids in mitochondria and/or peroxisomes. Hepatocytes oxidize fatty acids to form acetyl-CoA that can subsequently be used for ketone body production. Defects in fatty acid oxidation are therefore routinely detected by acylcarnitine spectrum analysis. Determination of hepatic and plasma acylcarnitine profiles revealed accumulation of medium- and long-chain acylcarnitines (C6-C18) in both compartments of LRH-1-KD mice compared to wildtype mice (Figure 2B), suggestive of incomplete oxidation of fatty acids. An increase in plasma non-esterified fatty acid (NEFA) levels was also observed in LRH-1-KD mice (Table 1, Figure 2C), suggesting that decreased hepatic oxidation results in their accumulation in plasma. Moreover, β-hydroxybutyrate, a marker of fatty acid oxidation and subsequent ketogenesis in liver, was decreased in plasma of LRH-1-KD mice under fed and fasted conditions (Table 1, Figure 2D). To directly assess hepatic fatty acid oxidation, *ex vivo* myristic acid oxidation was measured in primary hepatocytes isolated from wildtype and LRH-1-KD mice by assessing the rate of conversion of [9,10-^{3}H] myristic acid into $^{3}H_2O$.

Primary hepatocytes isolated from LRH-1 KD mice indeed showed decreased rates of β-oxidation compared to wildtype hepatocytes (Figure 2E).

To evaluate whether other changes in lipid metabolism could contribute to the development of hepatic steatosis, the hepatic fatty acid profile was analyzed. In addition to accumulation of non-essential fatty acids, LRH-1-KD mice exhibited increased concentrations of the essential fatty acids linoleic acid (C18:2ω6) and linolenic acid (C18:3ω3) (Figure 3A) which cannot be synthesized *de novo* and are hence derived from the diet. The expression of several genes involved in fatty acid synthesis such as *Lxra*, *Srebp1a*, *Srebp1c*, and *Acc1* remained unchanged (Figure 3B), whereas the expression of S*cd1* and the LRH-1 target gene *Fas* was even decreased. Assessment of *de novo* lipogenesis by MIDA [31], revealed no differences in lipogenesis between wildtype and LRH-1-KD mice (Figure 3C), which is consistent with the observed location of fat deposition, as no fat accumulation was observed in the perivenous zone, where lipogenesis occurs.



Supplemental figure 2. Triglyceride and cholesterol distribution in plasma lipoproteins is unchanged. Pooled plasma samples were fractionated by FPLC and triglycerides (A) and total cholesterol (B) levels were found to be unchanged in collected fractions of LRH-1-KD versus control mice.

Hepatic export of very low-density lipoprotein (VLDL) particles was not different between LRH-1-KD and control mice (Figure 3D). The mRNA expression levels of apoprotein B100 (*apoB*) and microsomal triglyceride transfer protein (*Mttp*), both essential for VLDL synthesis, remained unchanged in livers of LRH-1-KD mice (data not shown). In addition, total plasma TG concentrations and TG distribution in plasma lipoproteins were found to be unchanged between wildtype and LRH-1-KD animals (Table 1, Figure S2). Finally, total intestinal fatty acid absorption was calculated and found to be not different between wildtype and LRH-1-KD mice (data not shown).

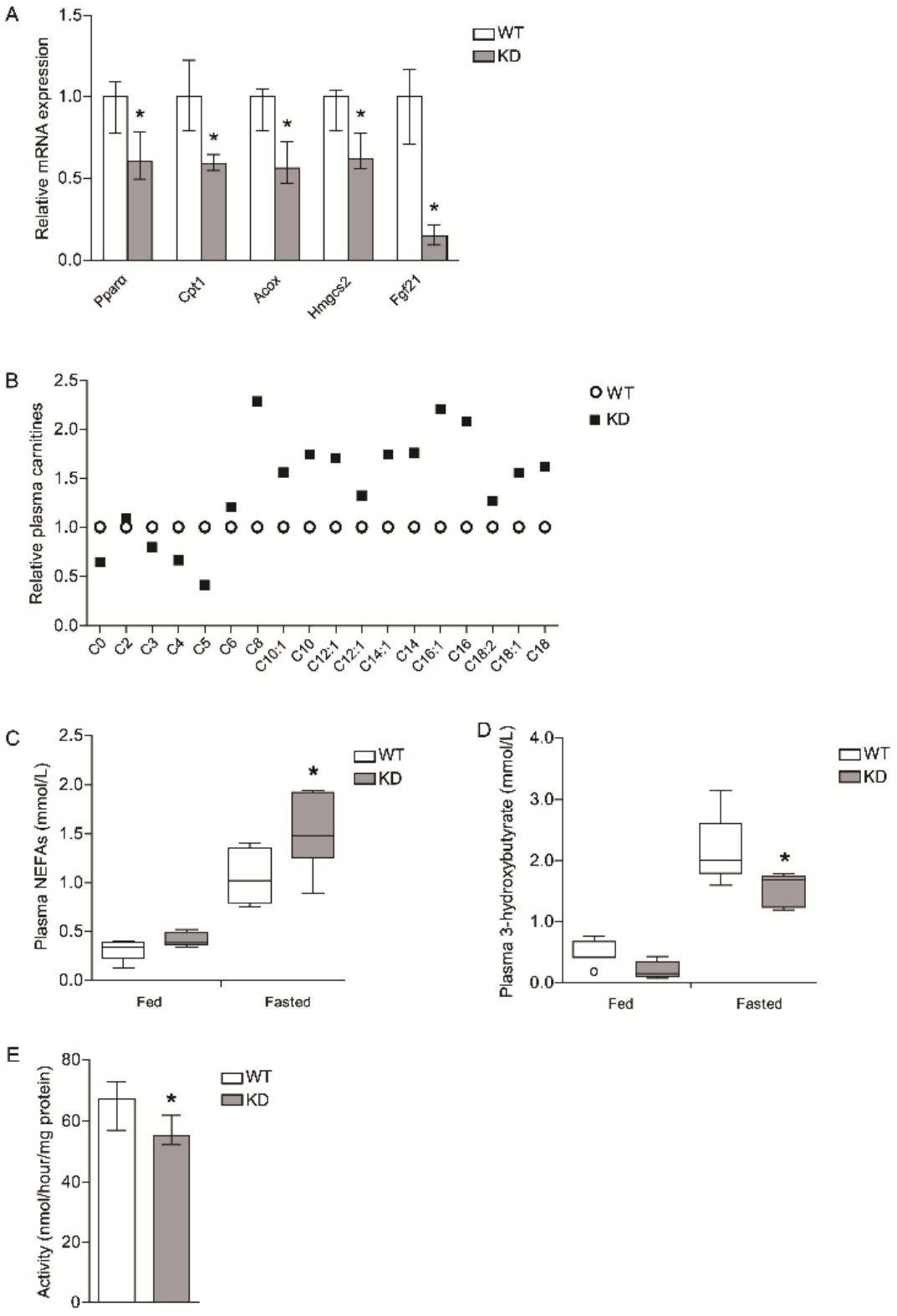


Figure 2. Decreased β-oxidation in LRH-1-KD animals. (A) Decreased gene expression of Pparα and its target genes is in LRH-1-KD mice. (B) Relative levels of medium- and long-chain acylcarnitines are increased in plasma of LRH-1-KD mice. Plasma NEFA levels (C) are increased, whereas 3-hydroxybutyrate levels (D) are decreased in fasted LRH-1-KD mice. (E) Ex vivo [9,10-3H] myristic acid oxidation is decreased in primary hepatocytes isolated from LRH-1-KD mice compared to wildtype mice.

Taken together, these data indicate that hepatic fat accumulation in LRH-1-KD mice is not due to alterations in either *de novo* synthesis of fatty acids, triglyceride export in VLDL particles or increased intestinal lipid absorption, but rather to suppressed β-oxidation capacity.

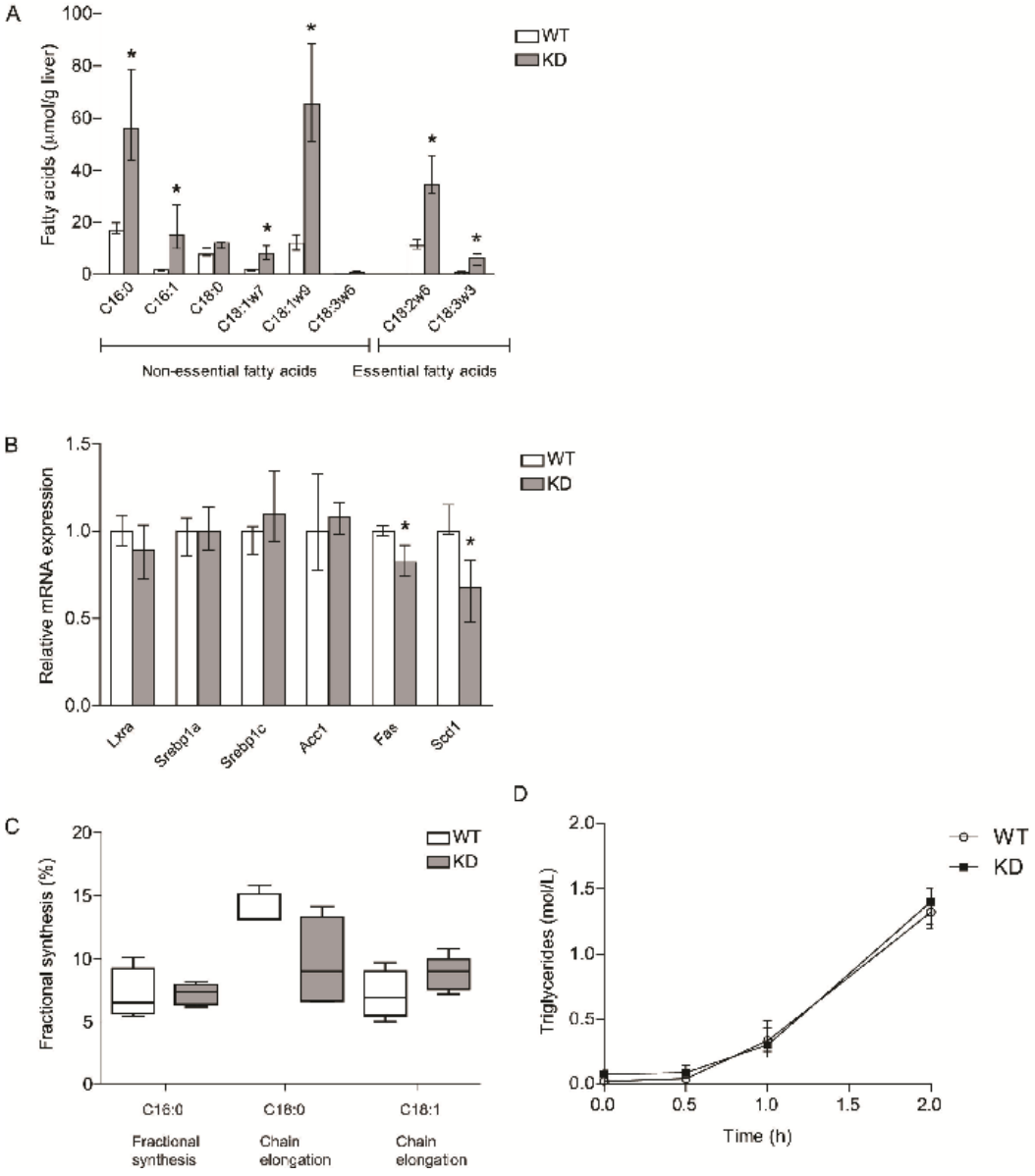


Figure 3. De novo lipogenesis and VLDL production are unaltered in LRH-1-KD mice. (A) Accumulation of both essential and non-essential fatty acids in livers of LRH-1-KD mice. (B) Expression of genes involved in de novo lipogenesis is unaltered or decreased in LRH-1-KD mice. (C) De novo lipogenesis measured by MIDA analysis is unchanged in LRH-1-KD mice. (D) The rate of very low-density lipoprotein (VLDL) production by the liver is similar in LRH-1-KD and wildtype mice.

LRH-1 binds the LRH-1 site in the PPARα promoter and regulates its transcriptional activity

Whereas whole-body knockdown of *Lrh1* was shown to impair the expression of *Pparα* and its target genes, adenoviral-mediated overexpression of *Lrh1* in HepA1.6 cells consistently induced the mRNA expression of *Pparα* (Figure 4A). The effects of LRH-1 on fatty acid β-oxidation and ketogenesis *in vivo* and on PPARα and its target genes suggest that LRH-1 might regulate PPARα expression. Recently, Chong *et al.* [10] suggested a role for LRH-1 in lipid metabolism based on a non-biased genome-wide ChIP-seq approach on mouse liver. Using this ChIP-seq dataset, LRH-1 binding to the PPARα promoter was visualized. Several LRH-1 binding peaks were identified in the PPARα proximal promoter (-1500 bp upstream of the transcription start site)(Figure 4B). In contrast, no peaks were detected on the FGF21 promoter (Figure 4C).

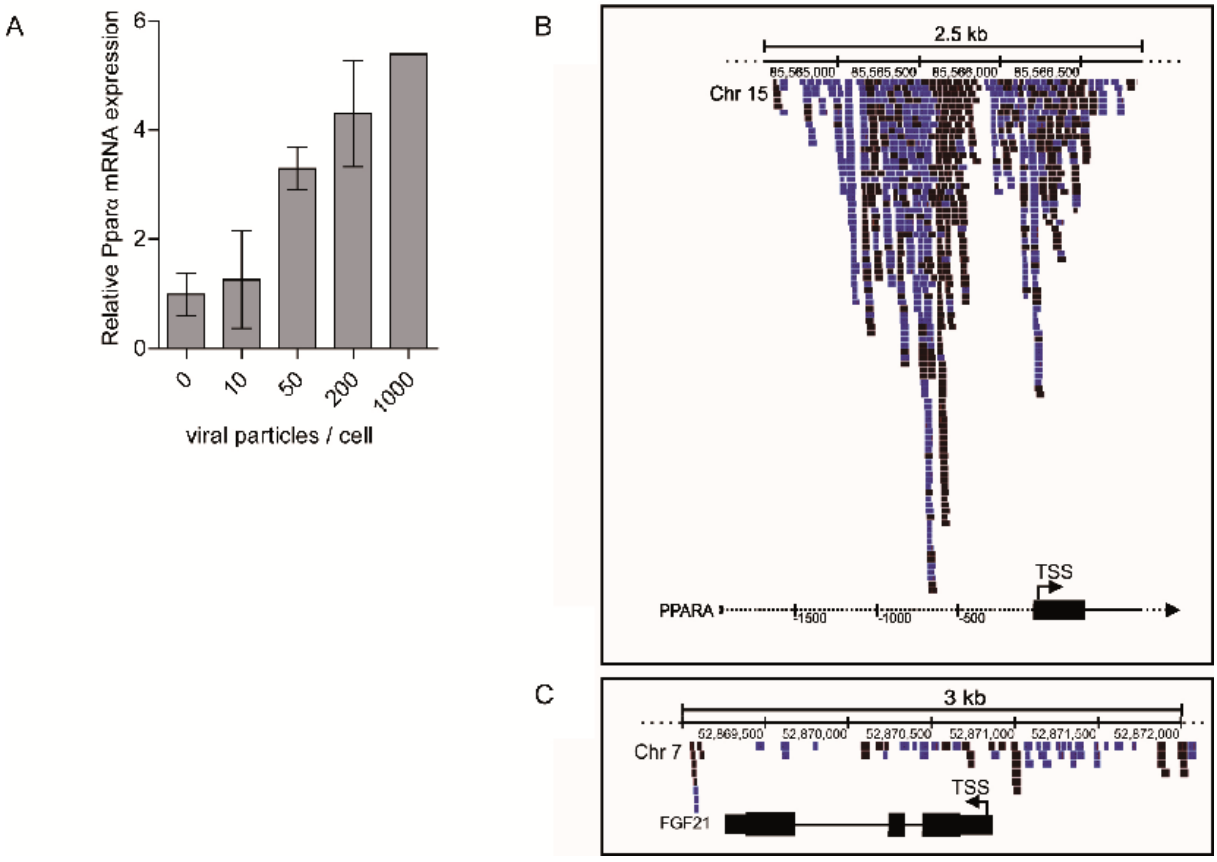
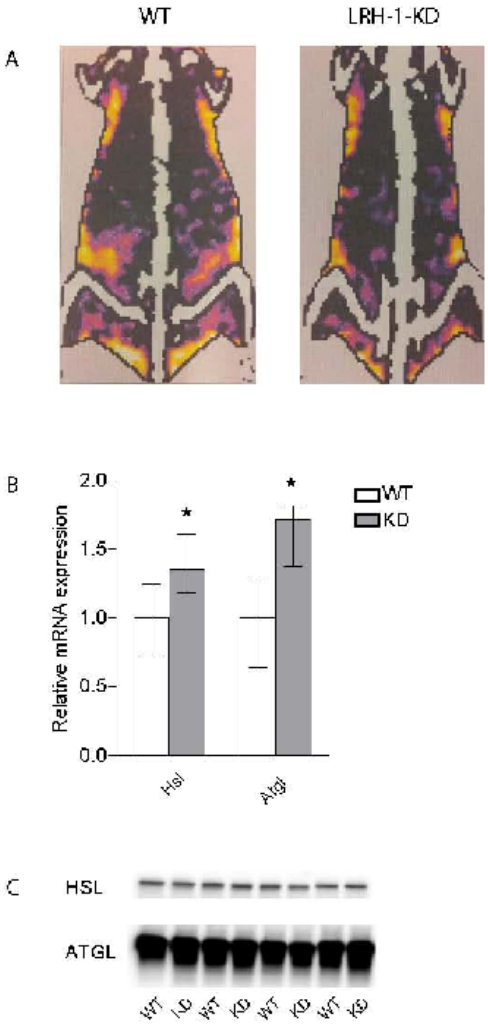


Figure 4. LRH-1 is recruited to the mouse PPARα promoter. (A) mRNA expression of Pparα is dose-dependently increased by adeno-LRH-1 transduction in HepA1.6 cells. Promoter regions of PPARα and FGF21 were inspected for the presence of LRH-1 ChIP-seq peaks visualized onto UCSC genome browser. (B) Representative view of ChIP-seq peaks on the mouse PPARα promoter. Promoter region and transcription start site (TSS) are indicated as described(50)(C) Representative view of ChIP-seq peaks on mouse FGF21 gene and promoter region. TSS is indicated as described(25)Shown are chromosomal locations according to the July 2007 Mouse Genome Assembly (mm9). Blue and red tags represent sequence reads from opposite DNA strands. The ability of LRH-1 to bind putative response elements in the PPARα gene was examined by EMSA as outlined in Materials and Methods.

Alignment of the mouse PPARα promoter sequence with the known LRH-1 consensus binding site identified several motifs with partial (-670, -861) or total (-571) homology with the consensus at -861, -670 and -571 base pairs upstream of the PPARα transcription start site (Figure 5A). To determine whether LRH-1 directly binds to those sites electrophoretic mobility shift assays were performed. Only the -670 and -571 sites specifically bound LRH-1 under these conditions with the -571 site displaying the strongest affinity for LRH-1 (Figure 5B). Interestingly, these two sites match with the strongest peak within the *Pparα* gene as determined by the LRH-1 ChIP-seq data analysis (Figure 4B). These strong protein-DNA complexes were efficiently competed by preincubation with increasing amounts of unlabeled wildtype probes, but not by the mutated oligonucleotides (Figure 5C). These results demonstrate that LRH-1 binds to the PPARα promoter *in vitro* and *in vivo*. Furthermore, a dose-dependent increase in transcriptional activity was observed when a luciferase reporter gene driven by the -926 to +131 sequence of the mouse PPARα promoter was co-transfected with increasing amounts of LRH-1 (Figure 5D). Thus, PPARα regulation by LRH-1 occurs at the transcriptional level.



Supplemental figure 3. LRH-1-KD mice have less white adipose tissue. (A) Representative DEXA-images of a wildtype (WT) and LRH-1-KD mouse show decreased white adipose tissue mass in the latter. (B) LRH-1-KD mice show increased expression of the lipolytic genes Hsl and Atgl in WAT. (C) HSL and ATGL protein levels in white adipose tissue are not different between wildtype and LRH-1-KD mice.

Lrh1 knockdown mice show a decrease in white adipose tissue mass

Compared to wildtype mice, LRH-1-KD mice show decreased gonadal white adipose tissue mass (Table 1). Furthermore, total body fat content measured by DEXA scanning was decreased in these mice (Figure S3A). In line with these results, mRNA expression of hormone-sensitive lipase (*Hsl*) and adipose triglyceride lipase (*Atgl*) was increased in WAT isolated from LRH-1-KD mice (Figure S3B). However, HSL and ATGL protein levels were not changed in the knockdown animals (Figure S3C).

DISCUSSION

Nutrient-sensing transcription factors play key roles in the maintenance of organismal energy homeostasis and are active in complex networks. In this study, we show that the expression of the pivotal transcription factor LRH-1 in livers of a large cohort of patients with NAFLD, correlated negatively with the extent of steatosis as well as with the severity of the necroinflammatory changes and fibrosis in NASH. Using an inducible knockdown mouse model, we subsequently investigated the molecular mechanism by which LRH-1 may control hepatic triglyceride metabolism. We identified PPARα to be an important bona fide downstream target of LRH-1 in the liver. LRH-1 is able to directly bind and activate PPARα, which is known as a major regulator of hepatic fatty acid metabolism, particularly during fasting: its actions promote uptake, utilization and catabolism of fatty acids and ketogenesis. FGF21 is a downstream target of PPARα that also stimulates hepatic fatty acid oxidation, ketogenesis and energy metabolism [26-28, 30, 32]. In LRH-1 KD mice the expression of *Fgf21* was decreased, however plasma levels were unchanged. Since no promoter binding was detected from ChIP-seq data [10], decreased expression is more likely to represent an indirect effect via PPARα. The reduction in PPARα signaling in LRH-1-KD mice resulted in decreased fatty acid oxidation and suppressed ketogenesis, leading to the development of hepatic steatosis because excess free fatty acids are stored as triglycerides. These results are in line with recent data showing that activation of LRH-1 by the phospholipid 1,2-dilauroyl-sn-glycero-3-phosphatidylcholine (DLPC; C12:0/C12:0) has anti-steatotic effects[11]. Upregulation of inflammatory responses in LRH-1-KD mice, as evident from microarray data analysis, might contribute to progression of hepatic steatosis to NASH, which is in line with the human data.

Since we used a conditional whole-body *Lrh1* knockdown mouse model, the contribution of decreased *Lrh1* expression in other tissues to the overall phenotype cannot be ruled out. LRH-1-KD mice show decreased white adipose tissue mass and increased plasma NEFA levels. However, in WAT *Lrh1* expression is very low compared to expression in the liver or the intestinal tract. The expression of *Hsl* and *Atgl* in WAT was increased, but protein levels were not different (Figure S3). This suggests that - at least at the time of sacrifice - enhanced lipolysis was not present. We cannot rule out that this occurred at an earlier time point leading to decreased levels of WAT, thereby increasing the lipid load on the liver and exacerbating hepatic fat accumulation. Previously we have shown that LRH-1-KD mice exhibit decreased expression of intestinal *Fgf15* [7], which may contribute to the development of a fatty liver. It has been shown that the human ortholog FGF19 influences hepatic beta-oxidation [33, 34]. However, also plasma TG-raising effects of FGF19 have been shown [35], possibly through different FGF receptors and target tissues. The exact role of FGF15/19 in lipid metabolism therefore remains to be determined.

Previously, two hepatic *Lrh1* knock-out models have been studied and showed no major differences in hepatic triglyceride content [6, 8]. Apart from differences in background strain which may influence the phenotype, we speculate that complete knock-out of a gene, even when organ specific, induces a much more pronounced compensatory effect of adjacent network genes which may partly rescue the phenotype. Indeed, surprising differences were seen between embryonic and inducible *Lrh1* knock-out mouse models. For example, *Lrh1* deficiency in livers of albumin-Cre mice did not significantly alter *Cyp7a1* mRNA levels, whereas acute knock-out of hepatic *Lrh1* in *Lrh-1fl/fl* mice did decrease basal *Cyp7a1* mRNA

levels[36] and conditional *Lrh1* knockdown mice could not upregulate *Cyp7a1*[7]. Moreover, *Lrh1*$^{+/-}$ mice show a remarkable distinct phenotype [37, 38], whereas (conditional) systemic disruption of *Lrh1* is lethal [1]. Thus, we speculate that due to its vital importance, chronic LRH-1 depletion leads to important compensatory regulation of adjacent network genes.

Given our results that LRH-1 knockdown leads to accumulation of hepatic triglycerides, we hypothesized that liver-specific *Lrh1* overexpression might protect against high fat diet-induced steatosis. Wildtype mice fed a high fat diet were injected with PBS or a self-complementary adeno-associated virus containing either the murine *Lrh1* gene, or GFP as a control. Unfortunately, only a 3-fold induction of hepatic *Lrh1* mRNA expression could be achieved without substantial changes in the expression of well-known target genes, whereas GFP was observed (using fluorescence microscopy) in 80% of hepatocytes transfected with scAAV-GFP (data not shown). Apparently, liver *Lrh1* expression is tightly regulated by feedback control mechanisms, which complicates overexpression experiments *in vivo*.

Hepatic LRH-1 is essential for the expression of CYP8B1, a key enzyme in the synthesis of the primary bile acid species cholic acid (CA). Hence, depletion of liver *Lrh1* decreases the contribution of CA-derived bile acids to the bile acid pool [6-8]. It has recently been reported that secondary bile acids, in particular deoxycholic acid (DCA) that is derived from CA, can have impact on hepatic triglyceride metabolism by potently inhibiting the NEFA importer protein FATP5[39] in the liver. Furthermore, 12-hydroxylated bile acids such as CA and DCA have been linked to dyslipidemia [40], providing another site of interaction between LRH-1 and triglyceride homeostasis. In addition, Chong *et al.* suggested that LRH-1 recruits the bile acid activated nuclear receptor FXR to lipid metabolic genes, thereby regulating genes of lipid metabolism in concert with FXR[10]. Alterations in FXR activity may therefore also contribute to the observed phenotype.

To ensure energy supply during alternating periods of fasting and feeding, hepatic lipid and carbohydrate metabolism are tightly synchronized [41, 42]. Several nuclear receptors, such as PPARα, show a strong circadian expression pattern [43, 44] and may link nutrient sensing (a.o. by fatty acids) to circadian control of metabolism. Coordinated regulation of both lipid and bile acid metabolism, couples intake of lipid substances to synthesis and secretion of bile acids to ensure proper lipid solubilization in the intestine. Because LRH-1 interacts with different nuclear receptors, transcription factors, including PGC-1α, SHP and SIRT1[3, 45, 46, 46-49] and target genes involved in lipid and bile acid metabolism, it may be a key component of the coordinated response necessary to relay circadian signals into metabolic responses.

It should be noted that although there is a striking concordance between LRH-1 expression and hepatic steatosis in mice and man, there are also differences. The mice show a decrease in WAT, humans obviously not. Another interesting difference is that the steatosis in mice is periportal whereas steatosis in humans is mostly pericentral. Importantly, however, the LRH-1-KD mice show upregulation of inflammatory pathways which is in line with the correlation between LRH-1 expression and the severity of NASH in the human cohort.

In conclusion, LRH-1 is a key player in the metabolic network controlling hepatic lipid homeostasis. In human liver LRH-1 is negatively correlated with NASH severity. Targeted activation of LRH-1 may therefore be beneficial and additional to fibrates in combatting

hepatic steatosis and its complications. On the other hand, when inhibiting LRH-1 as a potential target for contraception [50], lipid metabolic side-effects should be monitored.

EXPERIMENTAL PROCEDURES

Human study

Liver biopsies were obtained from patients visiting the obesity clinic of the Antwerp University Hospital, as recently described by Francque et al.(1) and analyzed by two experienced pathologist for histological features of NASH. The different histological features of NAFLD were assessed using the NASH Clinical Research Network (NASH CRN) Scoring System (2). The NASH Activity Score (NAS) was calculated by making the sum of the scores for steatosis, lobular inflammation and ballooning (2). The presence of NASH was defined according to Brunt et al.(3). LRH-1 mRNA levels were measured by QPCR as described above.

Animals

LRH-1 knockdown (LRH-1-KD) mice were obtained commercially from Taconic Artemis and described before(4). The model is based on a shRNA sequence targeting Lrh1 (NR5A2) cloned behind a doxycycline responsive promoter. Twenty to twenty-seven week old male or female LRH-1-KD mice on a C57BL/6J background and their wildtype littermates (WT) were housed in individual cages in a temperature- and light-controlled facility with 12 hours light-dark cycling. All mice, wildtype and transgenic, were fed commercially available laboratory chow (RMH-B; Hope Farms, Woerden, The Netherlands) supplemented with 200 mg/kg doxycycline for at least 4 weeks. DCL1 knockdown mice were obtained commercially from Taconic Artemis and were kindly provided by Dr. Saaltink (Leiden University, The Netherlands). Mice were terminated after a 4-hour fast at 10am, unless stated otherwise. All experiments were approved by the Ethical Committee for Animal Experiments of the University of Groningen.

Genotyping

Genomic DNA was extracted from ear lysates using REDExtract-N-AmpTM Tissue PCR Kit (Sigma-Aldrich, USA) and genotyping was performed as described before (4).

Gene array

Hepatic RNA was isolated using the RNeasy mini extraction kit (Qiagen # 74106) and the RNA integrity was verified by capillary electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA.). Double stranded cDNA was synthesized from 1.5 µg total RNA using the One-Cycle target Labeling Kit (Affymetrix Santa Clara, CA) and used for the preparation of biotin-labeled cRNA using the GeneChip IVT Labeling Kit (Affymetrix, Santa Clara, CA). Hybridization of cRNA and washing of Affymetrix GeneChip Mouse Genome 430 2.0 arrays was performed according to standard Affymetrix protocols. The arrays were laser scanned with a GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. Data was quantified using Microarray Suite 5.0/ GCOS 1.1 (Affymetrix). Genes were filtered that were present in more than two arrays, had a intensity of at least 20 in 1 of the arrays and showed a differential expression with a p-value lower than 0.05 and an adjusted p-value of 0.01. The gene expression dataset was

subsequently analyzed for gene ontology to relate changes in gene expression to functional alterations using the standard settings at the David webserver (5).

Hepatic TG export

To measure hepatic TG export, mice were fasted 4 hours prior to ip injection with 1000 mg/kg poloxamer407 (Pluronic® F127 NF Prill Poloxamer 407, BASF, Netherlands) to block the breakdown of lipoprotein particles (6). Blood samples were collected retro-orbital at 0, 30 minutes, 1 and 2 hours. The triglyceride export was calculated from the induction of plasma triglyceride levels two hours following P407 injection.

Plasma and Liver parameters

Liver histology was examined microscopically on paraffin embedded sections after Hematoxylin and Eosin (HE) staining by standard procedures. Hepatic lipids were extracted according to Bligh and Dyer(7). Hepatic fatty acid composition was analyzed by gas chromatography after transmethylation using C17:0 as an internal standard (8). Hepatic phospholipid content was determined as described previously (9). Plasma and liver triglyceride and cholesterol contents were determined using commercially available kits (Roche Diagnostics, Mannheim, Germany; DiaSys Diagnostic Systems, Holzheim, Germany). Plasma-free fatty acids were determined using a NEFA-C kit (Wako Chemicals, Neuss, Germany). Lipoprotein separation was performed on pooled plasma samples by fast protein liquid chromatography on a Superose 6 HR 10/300 GL column using an Akta Purifier (GE Healthcare, Diegem, Belgium). Plasma β-hydroxybutyrate concentrations were measured using standard procedure (Vitalab Selectra E, Merck, Germany). Plasma levels of alanine aminotransferase and aspartate aminotransferase were determined using commercially available kits (Spinreact) according to the manufacturer's protocol. Liver glycogen was determined as described (10). Plasma Fgf21 concentration was measured with an ELISA kit (R&D Systems Inc, Minneapolis, MN, USA).

Determination of de novo lipogenesis and chain elongation in vivo.

In order to measure lipogenesis in the liver, mice were given 2% labeled [1-13C] acetate in drinking water for 3 days. Liver homogenates were prepared and fatty acids were measured using a Agilent 5975series GC/MSD (Agilent Technologies, Santa Clara, CA) and the normalized mass isotopomer distributions were used in MIDA algorithms to calculate the acetyl-CoA precursor pool enrichment, fractional synthesis rate, and chain elongation rates (11).

Fat balance measurements

Fecal and food fatty acid composition was analyzed by gas chromatography after transmethylation and using C17:0 as internal standard as described (8). Total fat input or output was calculated as the sum of the molar amounts of each of the individual fatty acids multiplied by the total mass of food intake or feces per 24 hour.

RNA isolation and PCR procedures

RNA isolation en cDNA synthesis were performed as before (4). Gene expression was measured using quantitative polymerase chain reaction (qPCR) performed with a 7900HT FAST system using FAST PCR master mix, Taqman probes and MicroAmp FAST optical 96-well reaction plates (Applied Biosystems Europe, Nieuwekerk ad IJssel, The Netherlands). Primer and probe sequences are deposited at the RTPrimerDB (www.rtprimerdb.org). Relative expression levels were standardized to 36B4.

Isolation of primary hepatocytes

Primary hepatocytes were isolated a two-step collagenase perfusion protocol as described before (12, 13). Cell viability was determined by Trypan Blue exclusion. Directly after isolation, cells were plated on coated plates in William's E medium (Invitrogen, Breda, The Netherlands) supplemented with 50 µg/mL gentamycin (Invitrogen), penicillin–streptomycin, 50 nmol/L dexamethasone (Department of Pharmacy UMCG, Groningen, The Netherlands) and 0.5 ug/ml doxycycline. Cells were cultured in a humidified incubator at 37 °C and 5% CO2. Overall β-oxidation measurements using 3H-myristate. Whole cell beta-oxidation was measured in primary hepatocytes based on 3H2O-release using [9,10-3H] myristate as described(14).

Plasmids and cell transfection

AML12 cells (ATCC CRL-2254) were maintained in Dulbecco's Modified Eagle's Medium /Ham's Nutrient Mixture F-12 (1:1) with 2.5mM L-glutamine, 1.2g/L sodium bicarbonate, 15mM HEPES and 0.5mM sodium pyruvate supplemented with 10% fetal bovine serum, 5µg/ml insulin, 5µg/ml transferring, 5ng/ml selenium and 40ng/ml dexamethasone. For transient transfection cells were grown in 24-well plates. Seventy to eighty percent confluent cells were transfected using 2µL of jetPEI transfection reagent (Polyplus transfection), 40ng of pGL3basic (Promega) or pGL3mPPARα carrying the 926/+131 base pairs fragment of the PPARα promoter, 10ng of pRL- tk Renilla (Promega) and variable amounts (50, 100, 250ng) of pCMV6entry-mLRH-1 (Origene). Empty pBK-CMV expression vector (Stratagene) was used as a control and to maintain equal amount of DNA (1µg total DNA per well). Transfected cells were cultured in complete growth medium without dexamethasone. Luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) 48 hours after transfection. Transfections were carried out in triplicate, and the experiment was repeated twice.

Adenoviral transductions

Transductions were performed as described previously(4) at the indicated multiplicity of infection during 3 hours in medium without serum. Thereafter, cells received complete medium and were incubated for 24 hours and lysed for RNA isolation.

ChiP-Seq data analysis

The SAM ("SequenceAlignment/Map") file generated from ChIP-seq sequencing data to determine peaks containing binding sites of LHR-1 in mouse liver was kindly provided by Dr. Timothy F Osborne, University of California, USA. The SAM file was converted to the

compressed binary BAM format by the Galaxy SAM Tools(15) and visualized onto UCSC genome browser(16).

Electrophoresis Mobility Shift Assays

Mouse LRH-1 protein was translated in vitro using the TNT T7 Quick Coupled Transcription/Translation System (Promega). 30-nt double stranded oligonucleotides (-861: 5'-ccctggcaccttggccacctgttgccgcgt-3'; -670: 5'-gacccgcagccttgaacttcagtcctggcc-3' and -571: 5'-tcgggtgaccttgggcagtcccttcaccta-3') corresponding to the potential LRH-1 binding sites were used in EMSA. Two µL of rabbit reticulocyte lysate (RRL) expressing LRH-1 or unprogrammed RRL were mixed with 1 x 104 cpm of 32P-end-labeled double stranded oligonucleotides in a volume of 20µL of binding buffer (20mM HEPES pH 7.8, 7mM KCl, 1mM EDTA, 0.5mM EGTA, 5mM DTT, 5% glycerol, 0.05% NP-40, 5mM MgCl2, 0.3µg BSA, 2µg of dI-dC and 1X Complete Mini Roche Protease Inhibitor Cocktail). Reaction mixes were incubated for 15 min at room temperature, and protein-DNA complexes were resolved on 5% nondenaturing polyacrylamide gels run in 0.25X Tris-borate-EDTA at 150V for 2 hours at 4oC. For competition experiments, 2-, 5- and 25-fold molar excess of unlabeled competitor DNA relative to labeled DNA or mutated LRH-1 consensus binding site (mutant: 5'-tcgggtgaacctgggcagtcccttcaccta-3') were added to the reaction mix before the addition of the labeled probe.

Statistics

All values are presented as Tukey's Box-and-Whiskers plot using median with 25th to 75th percentile intervals (P25-P75) or bar charts with median +/- range. Plots were created using the GraphPad Prism 5 software package. Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, USA). Differences between the groups were analyzed by the non-parametric Mann Whitney U Test with $P < 0.05$ considered statistically significant. In human samples regression analysis was performed and Kruskal-wallis or one-way ANOVA tests were used.

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Chapter 7

SUMMARY AND PERSPECTIVES

In this thesis, the activities of PPARα both in physiological and pathological conditions have been broadly discussed. Mechanistically and functionally, hepatic PPARα is the major nuclear receptor controlling fatty acid turnover, adapting its transcriptional activity to maintain homeostasis in the fed state and during periods of starvation when PPARα triggers fatty acid oxidation and ketone body synthesis providing the source of energy for peripheral tissues [1].

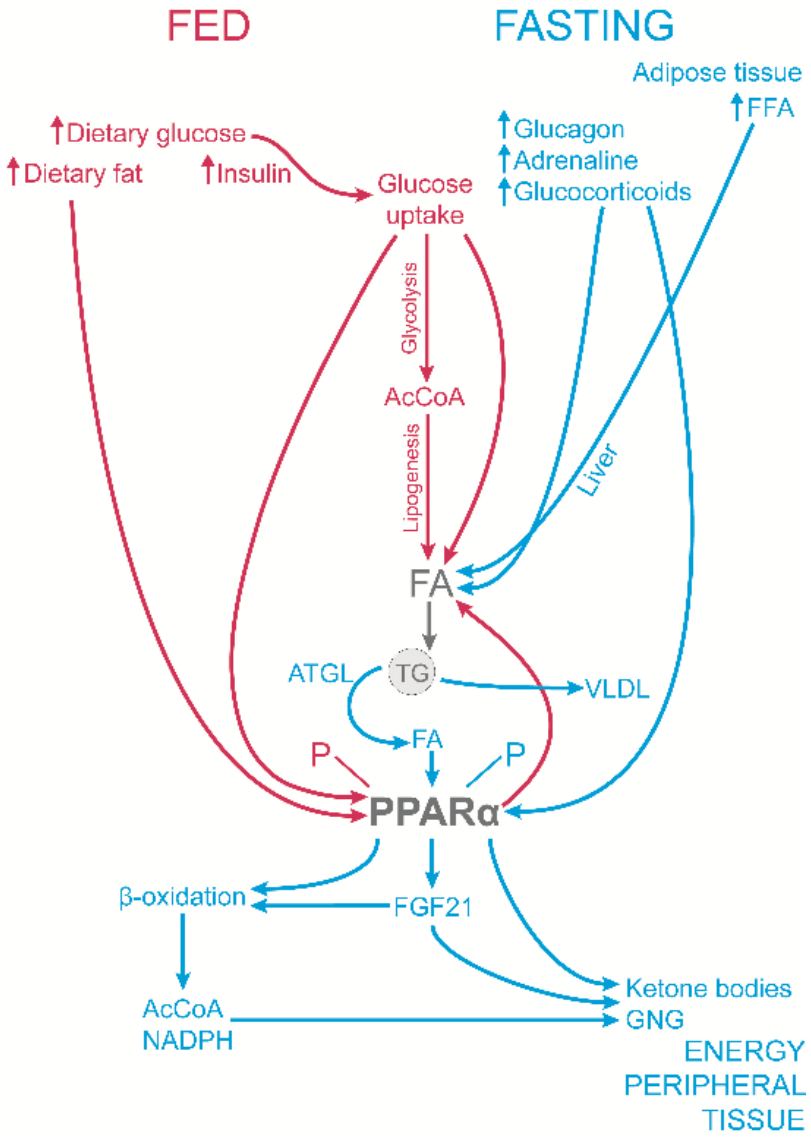

Figure 1. Molecular switch of PPARα activity in fed/fasted state. Augmented postprandial glucose levels lead to increased production and secretion of insulin by β-cells that induces glycolysis to yield acetyl-CoA (AcCoA) for further FA synthesis. Insulin stimulates PPARα phosphorylation and enhances its transcriptional activity. Additionally, lipogenesis yields fatty acid-derivatives operating as PPARα ligands. During fasting, stress hormones such as adrenaline and glucocorticoids are synthesized thus

in turn increase glucagon levels. Glucagon sustains gluconeogenesis through a stimulatory effect on the hepatic gluconeogenic precursor uptake as well as on the efficiency of gluconeogenesis (GNG) within the liver. The lipolytic release of adipose tissue fatty acids raises plasma levels of free fatty acids (FFA) that are subsequently stored in the liver as TG. ATGL-dependent hydrolysis of hepatic intracellular TG provides lipid ligands for PPARα activation. In fasting, PPARα activation leads to increased β-oxidation rates directly and via FGF21 activation to provide substrates for ketone body synthesis and GNG thus maintaining energy sources for peripheral tissues.

The importance of PPARα activity in different nutritional conditions is supported by the fact that natural PPARα ligands are formed either during lipogenesis or produced by lipid hydrolysis and fatty acid oxidation. However, a mechanism must exist to coordinate PPARα activation in response to different dietary signals. Recent studies show that FAS ability to synthesize phospholipids serving as endogenous ligands of PPARα stems from different subcellular localizations and posttranslational modifications of FAS [2]. Insulin-dependent phosphorylation of cytoplasmic FAS by target of rapamycin complex 1 (*mTorc1*) limits downstream generation of a PPARα ligand, whereas membrane-associated FAS, that produces lipids for energy storage and export, is less susceptible to phosphorylation. Conversely, in the fasting state, de-phosphorylated FAS in the cytoplasm is permissive for the generation of endogenous PPARα ligands thus activating PPARα-target genes [2].

The transcriptional activity of PPARα can also be directly regulated by phosphorylation e.g. p42/p44 Mitogen-Activated Protein Kinases (MAPK)-dependent phosphorylation [3] in response to insulin, stress-activated p38 MAPK phosphorylation [4] or PKC-dependent phosphorylation [5]. It has been proposed that a molecular switch exists to modulate PPARα activity by phosphorylation. Nevertheless, further studies should be performed to elucidate the importance of PPARα phosphorylation and different posttranslational modifications in physiologic conditions such as metabolic adaptation to fasting.

The essential role of PPARα in periods of starvation has been clearly demonstrated in *Pparα*$^{-/-}$ mice which show pronounced hypoglycemia and impaired ketone body levels after prolonged fasting. In line with that, a major part of PPARα target genes is related to fatty acid catabolism and ketogenesis (for more details see **chapter 3**). Moreover, transcriptomic data suggests that the hypoglycemia might be due to impaired gluconeogenesis caused by decreased expression of pyruvate carboxylase (*Pcb*), as well as lactate dehydrogenase A4 (*Ldh-a4*) [6]. However, impaired pyruvate dehydrogenase kinase, isozyme 4 (*Pdk4*) upregulation in *Pparα*$^{-/-}$ mice could abolish subsequent phosphorylation of the active form of the pyruvate dehydrogenase complex (*Pdc*), thereby increasing glucose utilization.

Besides its function in the molecular switch between the fed and fasting states, PPARα has been found to fulfil important role in several pathologies linked to impaired fatty acid turnover and inflammation. The most striking activity of pharmacologically-activated PPARα in humans is its ability to counteract hyperlipidemia by decreasing plasma TG and increasing plasma HDL levels [7]. These PPARα activities underline its role in preventing cardiovascular events in patients with type 2 diabetes mellitus (T2DM) and atherogenic dyslipidemia [8].

However, current studies show that beside its normolipidemic activities in pathological conditions, PPARα plays pivotal roles in the control of hepatic triglyceride homeostasis. As shown in **chapter 6** of this thesis, the *PPARα* gene can be a target of different nuclear receptors, such as LRH-1 which controls hepatic lipid turnover and ketogenesis via transcriptional regulation of PPARα, hence highlighting its prominent regulatory function in

fatty acid metabolism. These PPARα properties indicate that it can be a molecular target in NAFLD treatment, as this chronic liver disease is hallmarked by excessive lipid accumulation. The prevalence of NAFLD increases systematically and is associated with obesity and insulin resistance in most cases in Western world [9]. No pharmacotherapy is currently approved for NAFLD/NASH thus the potential usefulness of fibrates or other PPARα agonists should be considered as therapeutic approach.

Numerous studies performed in rodents show a beneficial role of PPARα agonism in the development of steatosis, steatohepatitis and liver fibrosis ([10] or **chapter 3**). Nevertheless, the exact effect of fibrates on NASH is not fully understood. It is hypothesized that pharmacological activation of PPARα leads to an increase in FAO rates thus clearing hepatic TG pools and improving steatosis [11]. This, in turn reduces toxic products of lipid peroxidation and ROS, as factors significantly contributing to the transition from benign steatosis to NASH [12]. The main objective of this thesis was to determine molecular mechanisms and the relative contribution of PPARα-driven transactivation vs. transrepression in controling of fatty acid metabolism and inflammation. Further, our efforts were concentrated to unravel the exact role of PPARα agonism in NAFLD progression.

We have created a PPARα mutant unable to bind PPREs ($PPAR\alpha_{DISS}$), and by applying different approaches shown that the lipid normalizing PPARα activities stem from its ability to positively regulated FAO gene transcription by a mechanism requiring PPARα interaction with PPREs (see **chapter 5**). Interestingly, we have demonstrated that ligand-activated PPARα hampers pro-inflammatory responses by DNA binding-independent interference with the NFκB and AP-1 signaling pathways. These PPRE-independent anti-inflammatory PPARα activities have been observed *in vitro* and *in vivo* in the LPS-induced endotoxemia model. We further explored the properties of this DNA-binding crippled PPARα in a model of chronic liver inflammation [methionine-choline deficient diet (MCDD)] which is histologically similar to human NASH In this dietary-induced steatohepatitis mouse model, hepatocyte-specific adeno-associated virus (AAV) reconstitution of PPARα expression showed that ligand-activated $PPAR\alpha_{DISS}$, despite its inability to decrease intrahepatic lipid accumulation, protects against MCDD-induced liver damage and inflammatory response, to an extent comparable to that of $PPAR\alpha_{WT}$. Surprisingly, MCDD-fed *Pparα*$^{-/-}$ mice exhibited progressive pericellular hepatic fibrosis that was markedly reversed by pharmacologically activated $PPAR\alpha_{DISS.}$

Altogether, the results presented in this thesis show that PPARα inhibits the transition from simple liver steatosis toward a pathological state of NAFLD and fibrosis through a mechanism independent of its effect on hepatic lipid turnover, shedding new light on the role of PPARα in the multiple-hit model of NAFLD progression.

However, further studies should be addressed to characterize the exact molecular mechanism of PPARα-dependent transrepression of pro-inflammatory and pro-fibrotic genes. Transcriptomic analysis of ligand-activated $PPAR\alpha_{DISS}$ in pro-inflammatory conditions combined with generation of a genome binding map of $PPAR\alpha_{DISS}$ should be performed to determine whether PPARα-driven gene transrepression occurs by a mechanism that requires interactions of PPARα with chromatin regulatory regions of PPARα agonist down-regulated genes, either indirectly or by DNA motifs different from PPREs (tethered transrepression). Nonetheless, it is also possible that transrepression by PPARα stems fully or partially from its direct protein-protein interactions with pro-inflammatory transcription factors such as AP-1 and NFκB, as previously proposed [13].

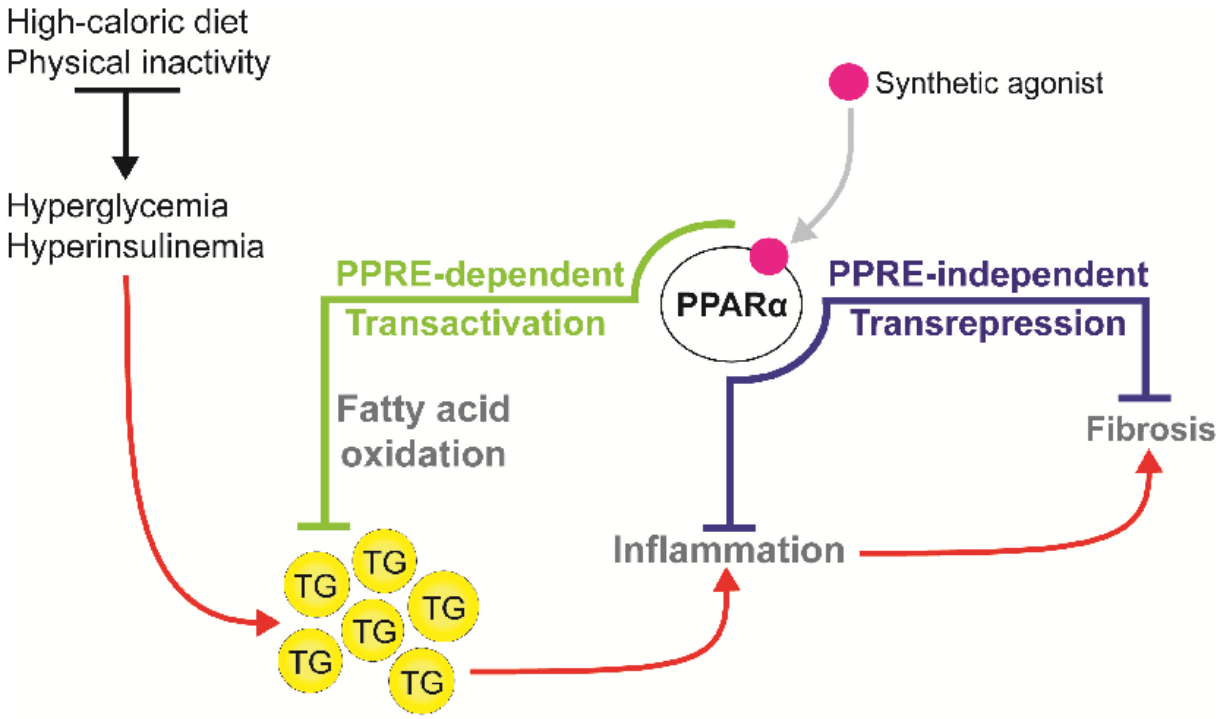

Figure 2. PPARα-dependent transactivation and transrepression in control of lipid utilization, inflammation and fibrosis in the liver. Excessive calorie intake and physical inactivity lead to development of liver steatosis. Accumulated hepatic TG are susceptible to lipoperoxidation that yields toxic compounds. Lipoperoxides and ROS induces hepatocyte damage that stimulates pro-inflammatory response. Chronic inflammation leads to fibrosis and liver scarring. As demonstrated by our study, PPARα may either counteract liver steatosis by PPRE-dependent mechanism resulting in transactivation of gene clusters related to FAO or decrease liver inflammation by PPRE-independent transrepression of pro-inflammatory and pro-fibrotic genes.

Altogether, the findings presented in this thesis highlight the potential of novel PPARα ligands in limiting the progression of chronic inflammatory liver diseases initiated by metabolic perturbations, through a direct counteraction of inflammatory responses independently of PPARα's effect on intrahepatic lipid accumulation. Selective and potent PPARα agonists with dissociated activity to trigger PPARα-dependent transrepression could thus be an option to treat inflammation and fibrosis concomitant NASH progression.

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AAV	adeno-associated virus
ABCD	ATP-binding cassette sub-family D
ACC1	acetyl-CoA carboxylase
ACOX1	acyl-CoA oxidase 1
AF-1	activation function 1
AF-2	activation function 2
ALA	alpha linolenic acid
ALMS1	Alstrom syndrome 1
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AP-1	activator protein 1
APO	apolipoprotein
APR	acute phase response
AR	androgen receptor
AST	aspartate transaminase
ATGL	adipose triglyceride lipase
BA	bile acid
BIEN	L-bifunctional enzyme
C/EBP	CCAAT-enhancer-binding protein
CA	cholic acid
CAR	constitutive androstane receptor
CAT	catalase
CBP	CREB-binding protein
CCL5/RANTES	chemokine (C-C motif) ligand 5
CD14	cluster of differentiation 14
CDS	coding DNA sequence
ChIP	chromatin immunoprecipitation
CHREBP	carbohydrate response element–binding protein
CoA	coactivator
COL1α1	collagen, type I, alpha 1
CoR	corepressor
COX-1	cyclooxygenase-1
CPT-1	carnitine palmitoyltransferase I
CRP	C-reactive protein
CTE	C-terminal extension
CVD	cardiovascular disease
CYP4A10	cytochrome P450, family 4, subfamily a, polypeptide 10
DBD	DNA-binding domain
DHA	docosahexanoic
DLPC	1,2-dilauroyl-sn-glycero-3-phosphatidylcholine
DR	direct repeat
ELOVL	fatty acid elongase
EMSA	electrophoretic mobility shift assay
EPA	Eicosapentaenoic acid
ER	estrogen receptor
eRNA	enhancer-templated non-coding RNA
ERR	Estrogen-related receptor
FABP	fatty-acid-binding protein
FAO	fatty acid oxidation
FAS	fatty acid synthase
FATP-1	fatty acid transport protein 1
FFA	free fatty acid
FGA	fibrinogen alpha
FGF21	fibroblast growth factor 21
FOXO1	forkhead box protein O1
FXR	farnesoid X receptor
GC	genome copy
GFP	green fluorescent protein
GPS2	G protein pathway suppressor 2
GR	glucocorticoid receptor
HAT	histone acetyltransferase
HDAC	histone deacetylase inhibitor
HDL	high-density lipoprotein

HEK293	human embryonic kidney 293 cells
HepG2	human hepatocellular carcinoma cells
HFD	high fat diet
HMGCS	3-hydroxy-3-methylglutaryl-CoA synthase
HSC	hepatic stellate cell
ICAM-1	intercellular adhesion molecule 1
IL-1β	interleukin 1 beta
IL-6	interleukin 6
NOS	nitric oxide synthase
IVC	inferior vena cava
KC	Kupffer cell
KD	ketogenic diet
LBD	ligand binding domain
LBP	ligand binding pocket
LCFA	long-chain fatty acid
LDL-C	low-density lipoprotein cholesterol
LPL	lipoprotein lipase
LPS	lipopolysaccharide
LRH-1	liver receptor homolog-1
LRH-1-KD	liver receptor homolog-1 knock down
LTB4	leukotriene B4
LXR	liver x receptor
MCD	methionine choline-deficient
MCDD	methionine choline-deficient diet
MED1	mediator of RNA polymerase II transcription subunit 1
MR	mineralocorticoid receptor
NAFLD	non-alcoholic fatty liver disease
NAS	NAFLD activity score
NASH	non-alcoholic steatohepatitis
NCoR	nuclear receptor co-repressor 1
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3	NOD-like receptor family, pyrin domain containing 3
NLS	nuclear localization signal
NR	nuclear receptor
nRE	negative response element
oxLDL	oxidized LDL
PGJ2	prostaglandin J2
PIC	pre-initiation complex
PKC	protein kinase C
PPAR	peroxisome proliferator-activated receptor
PPRE	PPAR response element
pRE	positive response element
PTE-2	peroxisomal acyl-CoA thioesterase 2
PUFA	polyunsaturated fatty acid
PXR	pregnane X receptor
RAR	retinoic acid receptor
ROR	RAR-related orphan receptor
ROS	reactive oxygen species
RXR	retinoid X receptor
SAA	serum amyloid a
SEC	sinusoidal endothelial cell
SF1	Steroidogenic factor 1
SFA	saturated fatty acid
SHP	small heterodimer partner
SIRT1	NAD-dependent deacetylase sirtuin-1
SMRT	silencing mediator for retinoid or thyroid-hormone receptors
SNP	single-nucleotide polymorphism

SRC-2	steroid receptor coactivator-2
SREBP-1c	sterol regulatory element-binding protein 1 c
T2DM	type 2 diabetes mellitus
TAA	thioacetamide
TBG	thyroxine binding globulin
TBL1	transducin beta-like protein 1
TBLR1	F-box-like/WD repeat-containing protein TBL1XR1
TFIIB	transcription factor II B
TG	triglyceride
TGFβ	transforming growth factor beta
TIMP1	tissue inhibitor of metalloproteinase
TLR	toll-like receptor
TNF	tumor necrosis factor
TSS	transcription start site
VCAM-1	vascular cell adhesion molecule 1
VDR	vitamin D receptor
VEGF	Vascular Endothelial Growth Factor
VLCAD	Very long-chain acyl-CoA dehydrogenase
ZF1	zinc finger 1
ZF2	zinc finger 2
16:0/18:1-GPC	1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine
8-LOX	8-lipoxygenase
8S-HETE	8(S)-hydroxyeicosatetraenoic acid