Chapter 4

The Role of Liver X Receptor in Hepatic *de novo* Lipogenesis and Cross-Talk with Insulin and Glucose Signaling

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1. Introduction

Regulation of nutrient balance by the liver is important to ensure whole body metabolic control. Hepatic expression of genes involved in lipid and glucose metabolism is tightly regulated in response to nutritional cues, such as glucose and insulin. In response to dietary carbohydrates, the liver converts excess glucose into fat for storage through *de novo* lipogenesis. The liver X receptors (LXRα and LXRβ) are important transcriptional regulators of this process. LXRs are classically known as oxysterol sensing nuclear receptors that heterodimerize with the retinoic X receptor (RXR) family and activate transcription of nutrient sensing transcription factors such as sterol regulatory element-binding protein 1c (SREBP1c) (Repa et al., 2000; Yoshikawa et al., 2002; Liang et al., 2002) and carbohydrate response element-binding protein (ChREBP) (Cha & Repa, 2007). LXR also induces the transcription of the lipogenic enzyme genes fatty acid synthase (FAS), stearoyl-Coenzyme A desaturase (SCD1) and Acetyl CoA carboxylase (ACC), alone or in concert with SREBP1c and/or ChREBP (Chu et al., 2006; Joseph et al., 2002; Talukdar & Hillgartner, 2006). LXR activate transcription of hepatic lipogenic genes in response to feeding, which is believed to be mediated by insulin (Tobin et al., 2002). The mechanisms by which insulin activates LXR-mediated gene expression is not clearly understood, but may involve production of endogenous ligand for LXRs and/or act by signal transduction mechanisms downstream of the insulin receptor (IR). Both glucose and insulin regulate *de novo* lipogenesis, however, some lipogenic genes can be regulated by glucose without the need of insulin which has been shown for SREBP1c (Hasty et al., 2000; Matsuzaka et al., 2004). A well known glucose-mediator in liver is ChREBP, an important regulator of *de novo* lipogenesis in response to glucose (Yamashita et al., 2001). ChREBP is activated by glucose via hexose- and pentose-phosphate-dependent mechanisms involving dephosphorylation of ChREBP and

translocation to the nucleus (Havula & Hietakangas, 2012). Interestingly, both LXR and ChREBP were recently shown to be post-translationally modified by O-linked β-N-acetylglucosamine (O-GlcNAc) in response to glucose potentiating their lipogenic capacity (Anthonisen et al., 2010; Guinez et al., 2011). Glucose flux through the hexosamine signaling pathway generates UDP-N-acetyl-glucosamine (UDP-GlcNAc), a substrate for O-GlcNAc modification of nucleocytoplasmic proteins by the enzyme O-GlcNAc transferase (OGT). We have shown that O-GlcNAcylation of LXR is increased in mouse livers in response to feeding and in livers from hyperglycemic diabetic mice potentiating SREBP1c expression (Anthonisen et al., 2010). Furthermore, preliminary studies in our laboratory indicate that LXR potentiate ChREBP activity under hyperglycemic conditions establishing a link between glucose metabolism, LXR and ChREBP. These observations suggest that LXR, SREBP1c and ChREBP contribute to converting carbohydrates into fat in a cooperative manner in response to high circulating glucose levels and that O-GlcNAc signaling plays a role in this process. As O-GlcNAc cycling appear to be essential for proper insulin signaling and the sensitivity of OGT to glucose increases with decreasing insulin signaling (Mondoux et al., 2011; Hanover et al., 2010) the relative roles of LXR, SREBP1c and ChREBP in regulating *de novo* lipogenesis in response to feeding and modification by O-GlcNAc signaling under insulin sensitive and insulin resistant conditions will be discussed.

2. Liver X Receptors (LXR)

2.1. LXR structure and function

LXRα (NR1H3) and LXRβ (NR1H2) are ligand-activated transcription factors belonging to the nuclear receptor (NR) superfamily (Lehmann JM (Lehmann et al., 1997; Willy et al., 1995; Janowski et al., 1996). LXRα is primarily expressed in metabolically active tissues, such as liver, intestine, adipose tissue, kidney and macrophages, whereas LXRβ is ubiquitously expressed (Apfel et al., 1994; Teboul et al., 1995; Teboul et al., 1995). LXRs are intracellular sensors of cholesterol and oxidized cholesterol derivatives (oxysterols) have been identified as their endogenous ligands (Janowski et al., 1996; Lehmann et al., 1997). The two isotypes originates from two different genes on separate chromosomes, but share the same modular structure, which is characteristic of most NRs (Fig. 1).

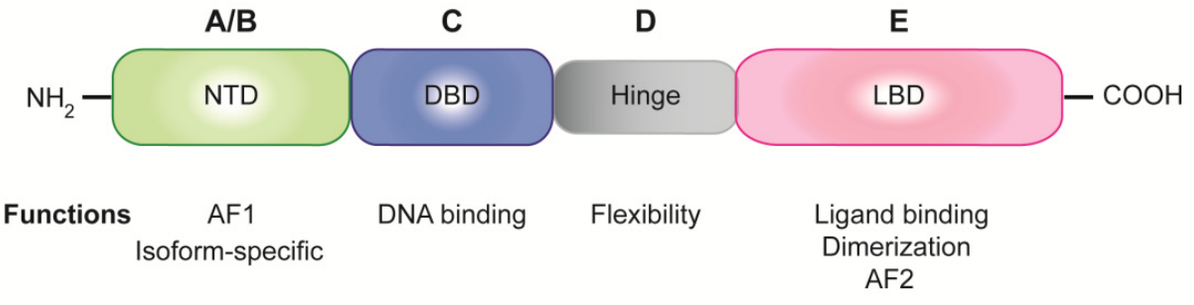

Figure 1. Structure of the LXRs

The DNA-binding domain (DBD) and the ligand binding domain (LBD) are highly structured domains. LXRα and LXRβ share 78 % amino acid sequence identity in these regions, while the N-terminal domain (NTD) and the hinge domain are far more disordered and less conserved. DNA binding requires dimerization with RXR. Transactivation by the LXRs is mediated through the ligand independent activation function (AF1) in NTD and the ligand dependent activation function 2 (AF2) in the LBD. Binding of a ligand to the hydrophobic ligand binding pocket leads to a conformational change that releases corepressors (CR) and exposes binding sites for coactivators (CA), recruiting the general transcription machinery and RNA polymerase II (RNA Pol II) (Fig. 2). This leads to changes in LXR dependent gene expression. The interactions with coregulators can also occur independently of ligand to AF1, however this is far less characterized. Upon activation, LXRs regulate a number of genes involved in lipid, cholesterol and glucose metabolism by binding to LXR response elements (LXREs) in their promoter region. These consist of a direct repeat of the nucleotide hexamer AGGTCA spaced by four nucleotides. Insights into LXR function in metabolism was provided by the generation of LXR mutant mice. These mice accumulate hepatic cholesterol, ultimately causing liver dysfunction (Peet et al., 1998; Ulven et al., 2005). It was found that LXRα controls cholesterol metabolism by conversion of cholesterol to bile acid by induction of the cholesterol 7 alpha-hydroxylase (Cyp7A1) gene, biliary cholesterol excretion and cholesterol efflux via induction of ABCG5/8 and ABCA1/ABCG1, respectively (Lehmann et al., 1997; Chiang et al., 2001; Yu et al., 2003; Repa et al., 2002; Graf et al., 2002; Costet et al., 2000; Sabol et al., 2005; Venkateswaran et al., 2000; Venkateswaran et al., 2000). LXRs are strongly implicated in the development of metabolic disorders and associated pathologies, notably, hyperlipidemia and atherosclerosis (Peet et al., 1998; Calkin & Tontonoz, 2010). Thus, LXRs are key players in maintaining metabolic homeostasis in health and disease by regulating inflammation and lipid/carbohydrate metabolism.

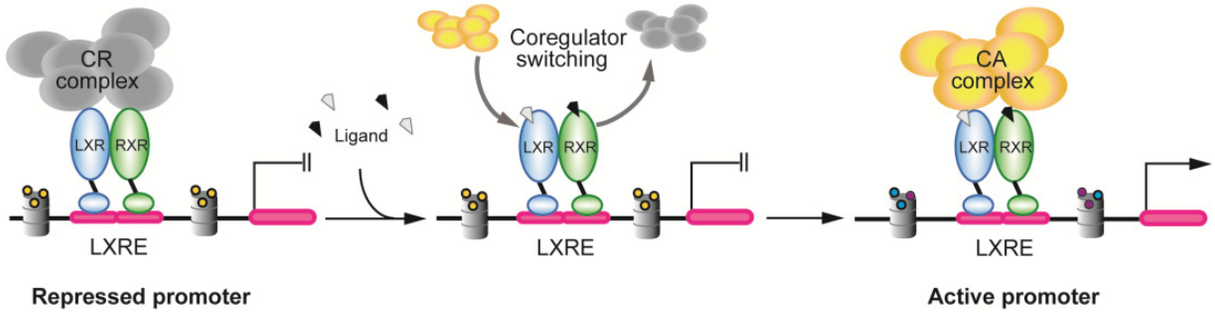

Figure 2. Activation of LXR by coregulator switching

2.2. Modulation of LXR activity by coregulators and PTMs

The transcriptional activity of LXRs is highly dependent on the presence of coregulators which has been linked to several metabolic processes (Jakobsson et al., 2009; Kim et al., 2003; Huuskonen et al., 2004; Kim et al., 2008; Oberkofler et al., 2003). Coregulators constitutes large multisubunit protein complexes containing chromatin-remodelling and/or –modifying

enzymes with intrinsic histone acetylase (HAT)/ deacetylase (HDAC) and histone methylase (HMT)/demethylase (HDM) activities, depending on whether they act as activators or repressors, respectively (Kato et al., 2011). It has been assumed that that the unliganded LXRs are localized in the nucleus and interact with CRs, including nuclear receptor corepressor/silencing mediator of retinoic acid and thyroid receptor (NcoR/SMRT) (Wagner et al., 2003). However, recent chromatin immunoprecipitation (ChIP) studies, including ChIP-sequencing (ChIP-Seq), have challenged this classical model. These studies put forward a more complex view, that ligands, pioneer factors, coregulators and posttranslational modifications (PTMs) play different roles in determining the LXR binding sites and actions *in vivo* (Boergesen et al., 2012; Heinz et al., 2010; Pehkonen et al., 2012). Furthermore, some coregulators have been shown to act as dual function activators/repressors, such as the coregulator protein receptor interacting protein 140 (RIP140). RIP140 has been shown to serve as a CA for LXR in lipogenesis but as a CR in gluconeogenesis independent of ligand activation (Herzog et al., 2007). General mechanisms of coregulator actions are assumed to be conserved between LXRs, but based on the low amino acid sequence identity in the NTD (32%) and the hinge domain (25%) it is possible that they contain novel isotype specific interaction surfaces. Also, the specific coregulator requirement to lipogenic LXR target genes in response to different feeding regiments under normal and diabetic conditions remain largely unexplored. In addition to ligand binding, LXRs can be posttranslationally modified by phosphorylation, acetylation, and sumoylation, affecting their target gene specificity, stability, and transactivating and transrepressional activity, respectively (Li et al., 2007; Ghisletti et al., 2007; Chen et al., 2006; Yamamoto et al., 2007). We have recently shown that LXR can be modified by O-GlcNAcylation in response to glucose (see section 4.3), increasing its transactivation of the SREBP1c promoter (Anthonisen et al., 2010). PTMs may alter the structural conformation of LXR thereby modifying the affinity of coregulators that determines whether a target gene is induced or suppressed. Modulation by PTMs can occur both in the absence and presence of natural ligand tuning LXR activities in a cell- and gene-specific manner (Rosenfeld et al., 2006) depending on the nutritional stimuli.

3. LXR in hepatic *de novo* lipogenesis

3.1. LXR lipogenic target genes

In addition to being central regulators of cholesterol metabolism, the LXRs are involved in induction of fatty acid and triglyceride (TG) biosynthesis in response to feeding. *De novo* lipogenesis ensures that excess acetyl-CoA, which is an intermediate product of glucose metabolism, is converted into fats and subsequent TGs. LXRs are involved in hepatic lipogenesis through direct regulation of SREBP1c and ChREBP expression (Repa et al., 2000; Cha & Repa, 2007; Shimano, 2001). SREBP1c is a well described transcriptional regulator of hepatic lipogenesis (Shimano, 2001), and together with LXR and glucose-regulated ChREBP (see section 4.1), it controls expression of essential enzymes in lipogenesis, lipid storage and secretion (Fig. 3). SREBP1c deficiency does not fully abolish the expression of genes involved

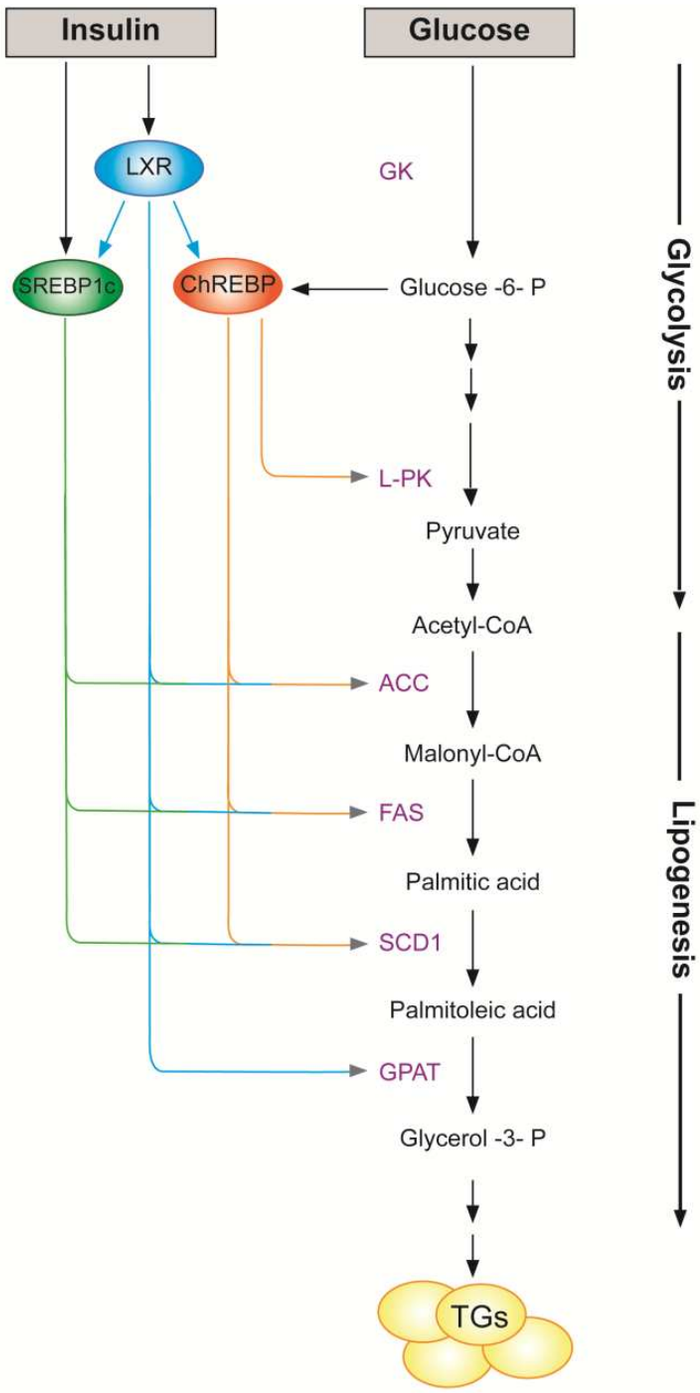

Figure 3. Regulation of hepatic lipogenesis by LXR, SREBP1c and ChREBP.

in hepatic lipogenesis. SREBP1c null mice treated with an LXR agonist results in induction of a subset of lipogenic genes and a modest increase in fatty acid synthesis (Liang et al., 2002), which implies that LXR can act independently of SREBP1c. In particular, the SCD1 gene is directly regulated by LXRα in response to synthetic ligands, also in the absence of SREBP1c (Chu et al., 2006). SCD1 is central in desaturation of saturated fatty acyl-CoAs important for formation of cholesterol esters (CEs) and TGs. Thus, specific LXR-mediated regulation of SCD1 can be explained by the essential role of LXR in limiting toxic free

cholesterol in response to diets rich in cholesterol and saturated fat. The expression of LXRα in liver is rapidly upregulated by insulin *in vivo*, increasing mRNA expression of SREBP1c, malic enzyme (ME), ACC and FAS. Furthermore, expression of these lipogenic genes was abolished in insulin-injected LXRα/β double knock out mice (Tobin et al., 2002), indicating an essential role for LXR in insulin-mediated regulation of hepatic lipogenesis. The mechanisms by which insulin activate LXR-mediated gene expression is not clearly understood, but may involve production of endogenous ligand for LXRα/β (Chen et al., 2004) and/or by signal transduction mechanisms downstream of the IR affecting CA recruitment to LXRs and/or PTMs of LXRs. This will be discussed in more detail below. Of note, PKA-induced phosphorylation of LXRα has been shown to inhibit the expression of SREBP1c in liver from mice via reduced DNA binding and CA recruitment (Yamamoto et al., 2007). Since glucagon/cAMP/PKA signaling may, at least in part, explain down-regulation of SREBP1c expression in response to fasting, it is likely that PKA-mediated phosphorylation of LXR contributes to the fasting signal on SREBP1c.

3.2. Putative mechanisms regulating LXR-mediated *de novo* lipogenesis in response to insulin

Insulin is the most important anabolic hormone in the body, regulating many processes important for cellular growth and energy storage such as glucose uptake and metabolism, glycogen and lipid synthesis, gene transcription and translation. A classic action of insulin is to mediate a metabolic switch from fatty acid oxidation to synthesis and suppress hepatic glycogenolysis and gluconeogenesis in response to carbohydrate excess, a process that is largely regulated at the transcriptional level. In this way, hepatic insulin signaling maintains whole body energy homeostasis. In the insulin-resistant state, only the ability of insulin to suppress hepatic gluconeogenesis is lost, while its ability to activate lipogenesis is retained (Shimomura et al., 2000; Matsumoto et al., 2006; Brown & Goldstein, 2008). This bifurcated insulin resistance can be explained by failure of insulin to inhibit the gluconeogenic transcription factor Forkhead box protein O1 (FoxO1), but maintaining signaling to lipogenic transcriptional regulators including LXR and SREBP1c.

3.2.1. The insulin signaling cascade

The insulin signaling cascade is initiated by the binding of insulin to the extracellular β-subunits of the dimerized IR followed by autophosphorylation on several intracellular tyrosine residues on the IR. Insulin receptor substrate (IRS) is an essential protein docking onto the phosphorylated IR which in turn is phosphorylated itself on multiple tyrosine residues. This creates docking sites for src homology 2 (SH2) domain containing proteins. The best studied SH2 protein that binds to tyrosine phosphorylated IRS proteins is the regulatory subunit of the phosphoinositide 3-kinase (PI3K). PI3K catalyzes the formation of the lipid second messenger phosphatidylinositol (3,4,5) trisphosphate (PIP3), which is necessary to recruit downstream kinases. PIP3 generates a binding site for proteins containing Pleckstrin homology (PH) domains, such as 3'-phosphoinositide-dependent

protein kinase (PDK1), the serine/threonine kinase Akt/protein kinase B and possibly also mammalian target of rapamycin complex 2 (mTORC2). PDK and mTORC2 are both necessary for full activation of Akt downstream of the insulin receptor via PDK1-mediated phosphorylation of Akt on threonine 308 and mTORC2-mediated phosphorylation on serine 472 (Saltiel & Kahn, 2001; White, 2003; Jacinto et al., 2006). All these events occur transiently in specific cholesterol rich plasma membrane microdomains called caveolae, generating a specific signaling unit for proper downstream insulin signaling where Akt plays a central role.

3.2.2. Regulation by mTOR

One of the targets of Akt is mTORC1 (Zoncu et al., 2011). Recent evidence suggests that mTORC1 is involved in LXR-mediated lipogenic gene transcription including induction of SREBP1c, FAS and ACC in liver from mice subjected to a high fat diet (Hwahng et al., 2009). The authors show that the mechanism by which mTORC1 activates LXR is via p70 S6 kinase (S6K)-mediated phosphorylation of LXR. Conversely, in the fasted state, LXR was shown to be inhibited by AMPK-mediated phosphorylation. In agreement with these observations, Li et al (Li et al., 2010) showed that insulin-activated hepatic transcription of SREBP1c, FAS and SCD1 is mediated by mTORC1, however independent of S6K. As both LXR and SREBP1c induce lipogenic promoters in response to insulin, this might suggest that activation of LXR in response to insulin/nutrients is mediated, at least in part, by mTORC1 and S6K, whereas insulin-signaling to SREBP1c requires mTORC2 independently of S6K, possibly via Akt-mediated inhibition of glycogen synthase kinase-3 (GSK3) (Hagiwara et al., 2012). In this way, GSK3-mediated phosphorylation and degradation of SREBP1c is prevented by insulin signaling to mTORC2 and Akt. Of note, insulin has primarily been shown to act on the SREBP1c promoter by activating LXRs and not SREBP1c (Chen et al., 2004) and the effect of insulin on SREBP1c is mainly at the posttranslational level. In a recent publication, mTORC1 was shown to phosphorylate a phosphatidic acid phosphatase, Lipin 1, preventing its nuclear entry and subsequent inhibition of SREBP1c-mediated activation of the FAS promoter (Peterson et al., 2011). Furthermore, Yecies JL et al (Yecies et al., 2011) showed that Akt2 independently of mTORC1 downregulate the mRNA expression of insulin induced gene 2 (Insig2a), an inhibitor of SREBP1c. This finding has been debated by Wan M et al (Wan et al., 2011), who could not observe any downregulation of Insig2a by Akt2. They postulate that Akt2 acts independently of mTORC1 and SREBP1c, possibly via posttranslational mechanisms, and that nutrients have a direct role in the liver to promote lipogenesis by a process dependent on both mTORC1 and other insulin-dependent signaling pathways. In light of the above mentioned studies, both mTORC1 and mTORC2 (Soukas et al., 2009; Guertin et al., 2006; Lamming et al., 2012; Hagiwara et al., 2012) appear to play important roles in lipid synthesis and storage in hepatocytes. Further studies will reveal the relative roles of Akt1, Akt2, mTORC1/C2 and S6kinase on activation of LXR and SREBP1c in this regulation under insulin sensitive and insulin resistant conditions and cross-talk with glucose metabolism and signaling (Fig.4).

3.2.3. Regulation by FoxO1

Another mechanism by which insulin may promote LXR-mediated SREBP1c transcription is through the transcription factor FoxO1. FoxO1, generally known as an activator of gluconeogenic genes during fasting, can repress the transactivating ability of LXR and cooperating transcription factors SREBP1c and Specificity protein 1 (Sp1) to activate SREBP1c transcription during fasting (Liu et al., 2010; Deng et al., 2012). FoxO1 does not seem to bind directly to the SREBP1c promoter, but appears to act as a repressor through protein-protein interactions, possibly by recruiting CR proteins (Deng et al., 2012). Upon feeding, FoxO1 is inhibited by insulin via PI3-kinase activation and phosphorylation by Akt, which excludes phosphorylated FoxO1 from the nucleus via association with the 14-3-3 protein (reviewed in (Tzivion et al., 2011)). In this way, at least under insulin sensitive conditions, inhibition mediated by FoxO1 and associating CRs is relieved, enabling LXR, Sp1 and SREBP1c to activate the SREBP1c promoter in a cooperative fashion. Of note, an important role for the E-box transcription factor Upstream Stimulatory Factor (USF) in mediating insulin activation of the SREBP1c promoter has also been reported (Wong & Sul, 2010). The relative roles of LXR, SREBP1c and cooperating transcription factors in regulation of the SREBP1c promoter after high-carbohydrate feeding under normal and insulin resistant conditions and the role of FoxO1 in this process in insulin resistance is currently not known. Recently, the role of Akt as a central regulator of both gluconeogenesis, through inhibition of FoxO1, and lipogenesis, through activation of mTORC1/2 in hepatic insulin signaling, was debated as the insulin resistant phenotype of mice lacking hepatic Akt1/2 were normalized in mice with concomitant liver-specific deletion of FoxO1 (Lu et al., 2012). This work suggests that a major role for Akt as a metabolic regulator in response to insulin is largely to restrain FoxO1 activity, at least for suppression of liver glucose output.

3.2.4. Regulation by insulin-mediated oxysterol production

Considering the bifurcated nature of insulin resistance and the postulated central role of Akt in this process, a very recent work by Wu and Williams (Wu & Williams, 2012), put forward an interesting theory. They suggest that disturbance of a single molecule, NAD(P)H oxidase 4 (NOX4), is sufficient to induce the key harmful features of insulin resistance. NOX4 is activated upon IR activation, generating a transient burst of superoxide (O_2^-) and its byproduct H_2O_2. This enhances signal transduction by disabling enzymes in the protein-tyrosine phosphatase gene family. In this way, essential inhibiting enzymes in the insulin signaling cascade is blocked, notably the PI3K inhibitor PTEN and protein-tyrosine phosphatase-1B (PTP1B) (Wu & Williams, 2012). Intriguingly, NOX4 may also be the link between insulin signaling and production of oxysterol ligand for LXR, as NOX4 through its superoxide producing activity may mediate the production of oxygenated cholesterol. The evidence for this is that pharmacological inhibition of NOX4 blocked insulin-induction of SREBP1c mRNA in rat primary hepatocytes, even though phosphorylations upstream and downstream of mTORC1 remained responsive (Wu & Williams, 2012). Furthermore, NOX4 is transiently localized to caveolae (Han et al., 2012), possibly via recruitment to the IR, placing the enzyme in close proximity to cholesterol-rich areas of the plasma membrane. A

complete summary of putative mechanisms of insulin-mediated signaling to LXR, SREBP1c and lipogenesis is depicted in Fig. 4.

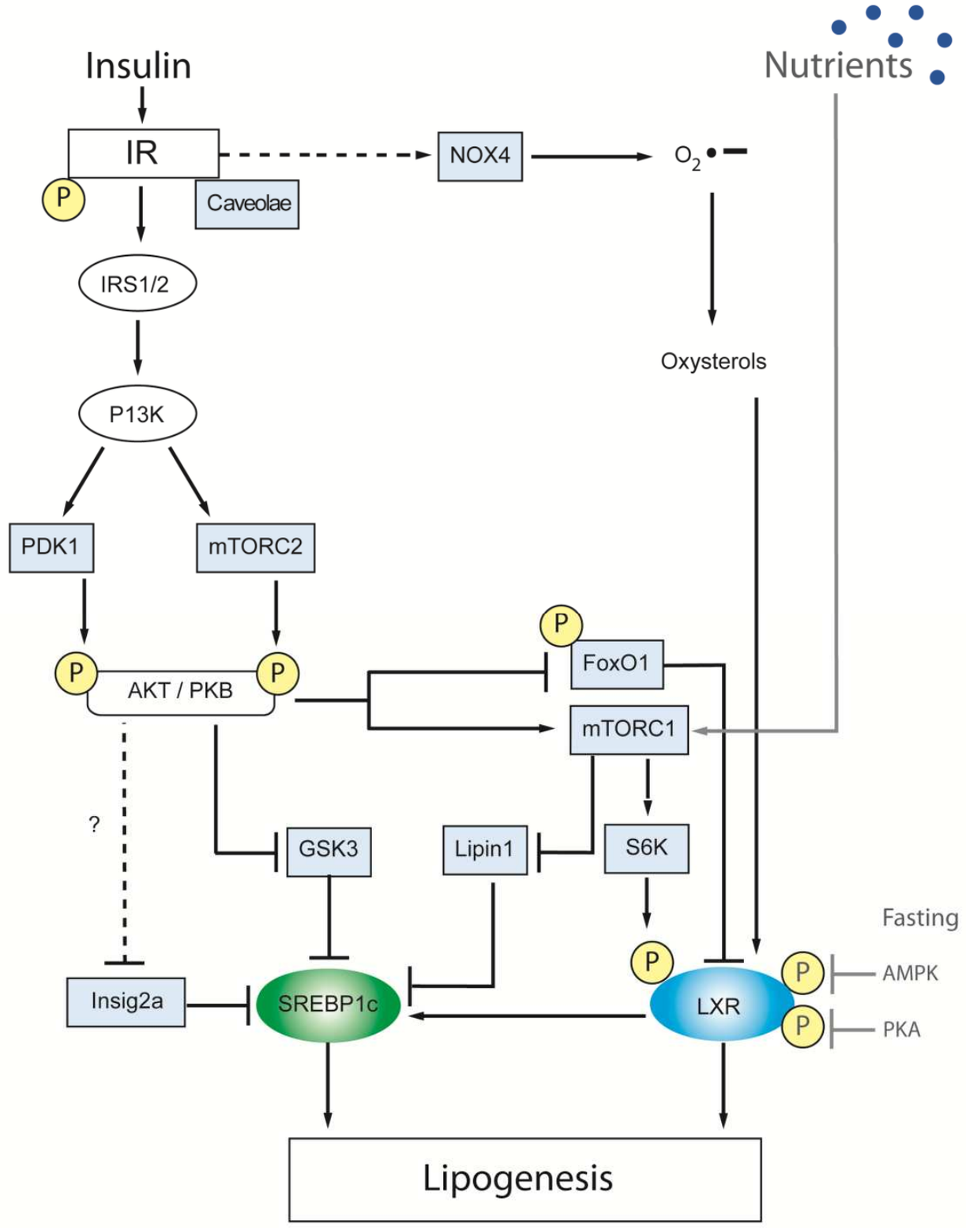

Figure 4. Insulin-mediated regulation of hepatic lipogenesis

4. Lipogenic gene expression in response to glucose metabolism

Hepatic glucose metabolism activates the transcription of various genes encoding enzymes of glycolysis and lipogenesis independently of insulin. However, the initial modification of glucose into Glucose-6-phosphate (G6P) by the enzyme Glucokinase (GK; Hexokinase 4) required for transcriptional regulation by glucose is highly dependent on insulin (Bosco et al., 2000), possibly via SREBP1c (Foretz et al., 1999; Kim et al., 2004) in concert with LXR and Peroxisome Proliferator-Activated Receptor gamma (PPARγ) (Kim et al., 2009). Thus the actions of glucose and insulin may be considered interdependent and that regulation of gene expression in response to glucose seems to require active LXR, SREBP1c and/or PPARγ.

4.1. Glucose regulation via ChREBP

A majority of hepatic glucose-responsive genes is thought to be regulated by the transcription factor ChREBP (Yamashita et al., 2001; Ishii et al., 2004). ChREBP mediates transcriptional regulation of glycolytic and lipogenic enzymes and is particularly important for the induction of liver-pyruvate kinase (L-PK), one of the rate limiting enzymes of glycolysis, which is exclusively dependent on glucose (Matsuda et al., 1990; Dentin et al., 2004). Furthermore, ChREBP is involved in regulating ACC and FAS in concert with LXR and SREBP1c in response to glucose and insulin, respectively, suggesting its involvement of the conversion of carbohydrates into fat (Joseph et al., 2002; Talukdar & Hillgartner, 2006). Moreover, stimulation by a synthetic LXR ligand, induces hepatic expression and activity of ChREBP (Cha & Repa, 2007). However, ChREBP is apparently not dependent on LXR for its hepatic expression and activity in mice fed a high carbohydrate/high fat diet (Denechaud et al., 2008), suggesting that ChREBP activity is reinforced by upstream LXR under certain nutritional conditions. At low glucose concentrations, the ChREBP protein is retained as an inactive phosphoprotein in the cytoplasm (reviewed in (Havula & Hietakangas, 2012)). The mechanisms by which glucose activate ChREBP is not clear, but involves induction of the ChREBP mRNA, dephosphorylation of the protein, shuttling to the nucleus and binding to the ChREBP response element at the promoter of its target genes (Uyeda & Repa, 2006). Early studies pointed to xylose 5-phosphate (Xu5P), an intermediate of the pentose phosphate pathway (PPP), as an activating signal through its ability to activate protein phosphatase 2A (PP2A) and subsequent dephosphorylation of ChREBP (Havula & Hietakangas, 2012). Recently, ChREBP was shown to be activated by fructose 2,6-biphosphate (F2,6BP) in hepatocytes (Arden et al., 2012). The level of F2,6BP is regulated by the bifunctional enzyme 6-phosphofructokinase-2-kinase/fructose-2,6-biphosphatase (PFK2/FBP2). Thus, PFK2 catalyzes the synthesis and degradation of F2,6BP and as a result, the enzyme is involved in both glycolysis and gluconeogenesis. In the fed state, insulin and carbohydrates dephosphorylate PFK2 in the liver making the enzyme kinase dominant. Subsequently, F6P is converted to F2,6BP that activates PFK1, which in turn stimulates glycolysis (Fig. 6). Interestingly, LXRα was recently shown to be a central regulator of hepatic PFK2 mRNA expression (Zhao et al., 2012). Activation of ChREBP in response to glucose appears to depend on multiple glucose metabolites, including G6P, X5P and F2,6BP. As LXRα

is involved in regulation GK- and PFK2-expression in response to insulin, this may suggest that ChREBP is dependent on insulin signaling via LXR for proper substrate availability.

4.2. Glucose metabolism via the hexosamine biosynthetic pathway and O-GlcNAc signaling

Glucose metabolism from F6P can follow the alternative hexosamine biosynthetic pathway (HBP) where the enzyme glutamine fructose-6-phosphate amidotransferase (GFAT) controls the first and rate limiting step (Fig. 5).

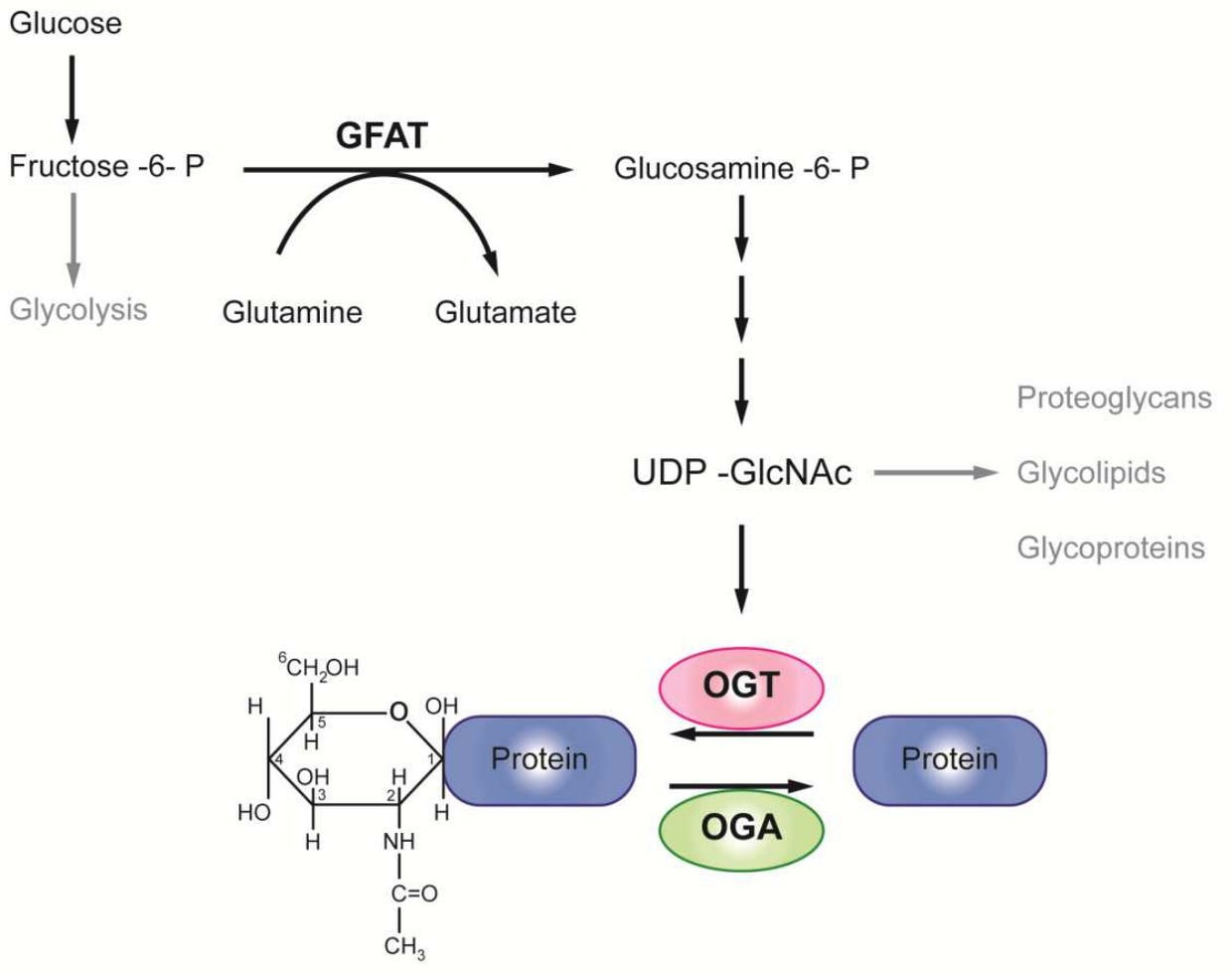

Figure 5. Nutrient flux and O-GlcNAc modification of nucleocytoplasmatic proteins through the HBP

The end product of this pathway is Uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc), an essential building block for N-and O-linked glycosylation of proteins and lipids. Cytoplasmic and nuclear proteins can be dynamically modified by O-linked β-N-acetylglucosamine (*O*-GlcNAc) on serine and threonine residues by the enzyme O-GlcNAc transferase (OGT) using UDP-GlcNAc as substrate. OGT is an essential enzyme as targeted deletion of this gene is lethal (Shafi et al., 2000). The enzyme O-GlcNAc transferase (OGA) hydrolyses the sugar analogous to protein dephosphorylation of phosphorylated proteins

by phosphatases (Hart et al., 2007; Love, 2005). Because *O*-GlcNAc levels on proteins appear to be sensitive to increasing flux through this pathway in response to nutrient excess, OGT can be considered as a general sensor of glucose availability that modifies proteins according to changes in UDP-GlcNAc levels. There is no identified consensus sequence for GlcNAcylation, and unlike the multiple genes encoding kinases, there is only a single X-linked gene encoding the catalytic subunit of OGT in mammals (Shafi et al., 2000). For this reason, it has been hypothesized that OGT is the catalytic subunit in large transient enzyme complexes where interacting proteins are able to target OGT to its many substrates. Many transcription factors are modified by O-GlcNAc in the liver (Dentin et al., 2008; Housley et al., 2008; Kuo et al., 2008; Ozcan et al., 2010). Interestingly, FoxO1 has been shown to be a target for O-GlcNAcylation in hepatocytes in response to hyperglycemia in the insulin resistant state, resulting in elevated transactivating capacity for FoxO1 against its gluconeogenic targets phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) reinforcing hepatic glucose production (Housley et al., 2008; Kuo et al., 2008). Moreover, this activation was later shown to be dependent on targeting of OGT to FoxO1 via interaction with the coactivator PGC1α, which itself was shown to be modified by O-GlcNAc upon interaction with OGT (Housley et al., 2009). As PGC1α have been shown to significantly amplify LXRα-mediated activation of the SREBP1c promoter (Oberkofler et al., 2003; Kim et al., 2008), a possible recruitment of an OGT/PGC1α-complex to LXR on lipogenic target genes under insulin resistant conditions remains to be explored. Recently, ChREBP was also shown to be a target for O-GlcNAcylation in response to hyperglycemia (Guinez et al., 2011). Adenoviral overexpression of OGT in liver increased ChREBP O-GlcNAc modification, protein stability and transactivating activity of L-PK, as well as potentiating expression of ACC, FAS and SCD1 mRNA expression in response to refeeding (Guinez et al., 2011). In contrast, hepatic overexpression of OGA reduced lipogenic protein content (ACC and FAS) and hepatic steatosis (excessive accumulation of TGs and CEs) in db/db mice, suggesting that enhanced OGT signaling to ChREBP and cooperating transcription factors/coregulators contributes to hepatic steatosis under insulin resistant conditions.

4.3. O-GlcNAc signaling activates LXR and hepatic lipogenesis

In 2007, glucose was reported as an endogenous ligand for LXR (Mitro et al., 2007). This has, however, been debated considering the hydrophobic nature of the ligand binding pocket (Lazar & Willson, 2007). Instead, we asked the question whether glucose exert its effect via hexosamine signaling and posttranslational O-GlcNAc modification of LXR. In a recent publication, we show that LXR is O-GlcNAc modified in response to high glucose (25 mM) in absence of insulin (cells cultured in 2 % serum, approximately 1-2 pmol/l insulin) and synthetic LXR-ligand in Huh7 cells, a human hepatoma cell line (Anthonisen et al., 2010). By pharmacological inhibition we demonstrated that hexosamine signaling and O-GlcNAc cycling mediates LXR dependent activation of the SREBP1c promoter in response to glucose. Furthermore, we observed increased O-GlcNAc modification of LXR in livers from refed mice and streptozotosin (STZ) treated diabetic mice corresponding with increased SREBP1c

mRNA expression. Moreover, general protein O-GlcNAcylation was increased in STZ-treated hyperglycemic mice compared to control mice. Our results suggest that LXR is regulated by O-GlcNAc modification, thereby increasing its lipogenic potential. Whether O-GlcNAc-LXR is able to transactivate other lipogenic genes in addition to SREBP1c, is currently under investigation in our laboratory. Our preliminary studies point to a role for O-GlcNAc-LXR in upregulating ChREBP, FAS, ACC and SCD1 expression (Bindesbøll et al, unpublished). Furthermore, preliminary reChIP experiments in our laboratory (LXR ChIP followed by O-GlcNAc ChIP), show a strong induction of O-GlcNAc-associated LXR binding to LXRE on the promoters of SREBP1c, ChREBP, FAS and SCD1 in response to feeding both in control mice and STZ treated mice. Our study is supported by the observation that the SREBP1c promoter activity and protein levels of SREBP1c are increased in response to elevated glucose concentration in the mouse hepatocyte cell line H2-35 (Hasty et al., 2000). Furthermore, treatment with azaserine, an inhibitor of GFAT, completely suppressed expression of both cytoplasmic and nuclear SREBP1c protein, suggesting that hexosamine-dependent O-GlcNAc signaling indeed is involved in glucose-induced SREBP1c mRNA expression, possibly via activation of LXR and/or cooperating transcription factors/CAs.

In our *in vitro* studies, we observed only modest LXR/RXR transactivation of the SREBP1c promoter in high glucose/low insulin-treated cells. This might be explained by constitutive phosphorylation competing for the same site(s) as GlcNAc on LXR and/or inhibitory phosphorylation occurring on adjacent GlcNAc sites. Housley et al. (Housley et al., 2008) reported elevated *O*-GlcNAc on FoxO1 by high glucose and a subsequent reduction by insulin. They further showed that *O*-GlcNAc modification increased substantially on the insulin-insensitive mutant FoxO1 lacking three AKT phosphorylation sites (T24A, S256A, S319A), resulting in increased FoxO1-dependent luciferase reporter activity. These observations imply overlapping and/or adjacent phosphorylation and GlcNAc sites on FoxO1. Indeed, the authors also identified several O-GlcNAc sites on FoxO1, one of which is adjacent to an Akt phosphorylation site (Thr^{317}). In the case of LXR, which is activated by insulin, apparently in part via S6K-mediated phosphorylation (Hwahng et al., 2009), GlcNAcylation and phosphorylation might act synergistically on LXR in response to glucose and insulin. In fact, extensive cross-talk between O-GlcNAcylation and phosphorylation appear to contribute to the pathology of various diseases (Hart et al., 2011). In addition, GlcNAc and inhibiting phosphate (in response to fasting via PKA and/or AMPK) may compete for the same sites or are situated at different serines and/or threonines on LXR. Furthermore, GlcNAcylation and phosphorylation of LXR might be affected by ligand binding, which has been shown for SUMOylation and acetylation of LXR (Venteclef et al., 2010; Lee et al., 2009). A study by Torra et al. (Torra et al., 2008) reported that Ser^{198} phosphorylation of LXRα in RAW macrophages was induced by both synthetic and natural oxysterol LXR ligands and reduced by the RXR ligand 9-*cis*-retinoc acid. As such, we cannot exclude the possibility that LXR O-GlcNAcylation may be positively or negatively regulated by LXR and/or RXR ligands. From our *in vitro* GlcNAcylation results (Anthonisen et al., 2010) we believe that the major O-GlcNAc site(s) on LXRα and LXRβ resides in the N-terminal region containing the AF1 and DBD, indicating that O-GlcNAcylation occur independently of ligand. However, under hyperglycemic conditions,

ligand binding may recruit OGT to LXR via CAs, possibly PGC1α as reported for FoxO1 (Housley et al., 2009). A more detailed mapping of the GlcNAc sites on LXR and site-directed mutagenesis as well as identification of coregulators of LXR under hyperglycemic conditions, are under way in our laboratory to elucidate the biological role of O-GlcNAc on LXR. A complete summary of putative mechanisms of glucose-signaling to LXR, ChREBP and lipogenesis is depicted in Figure 6.

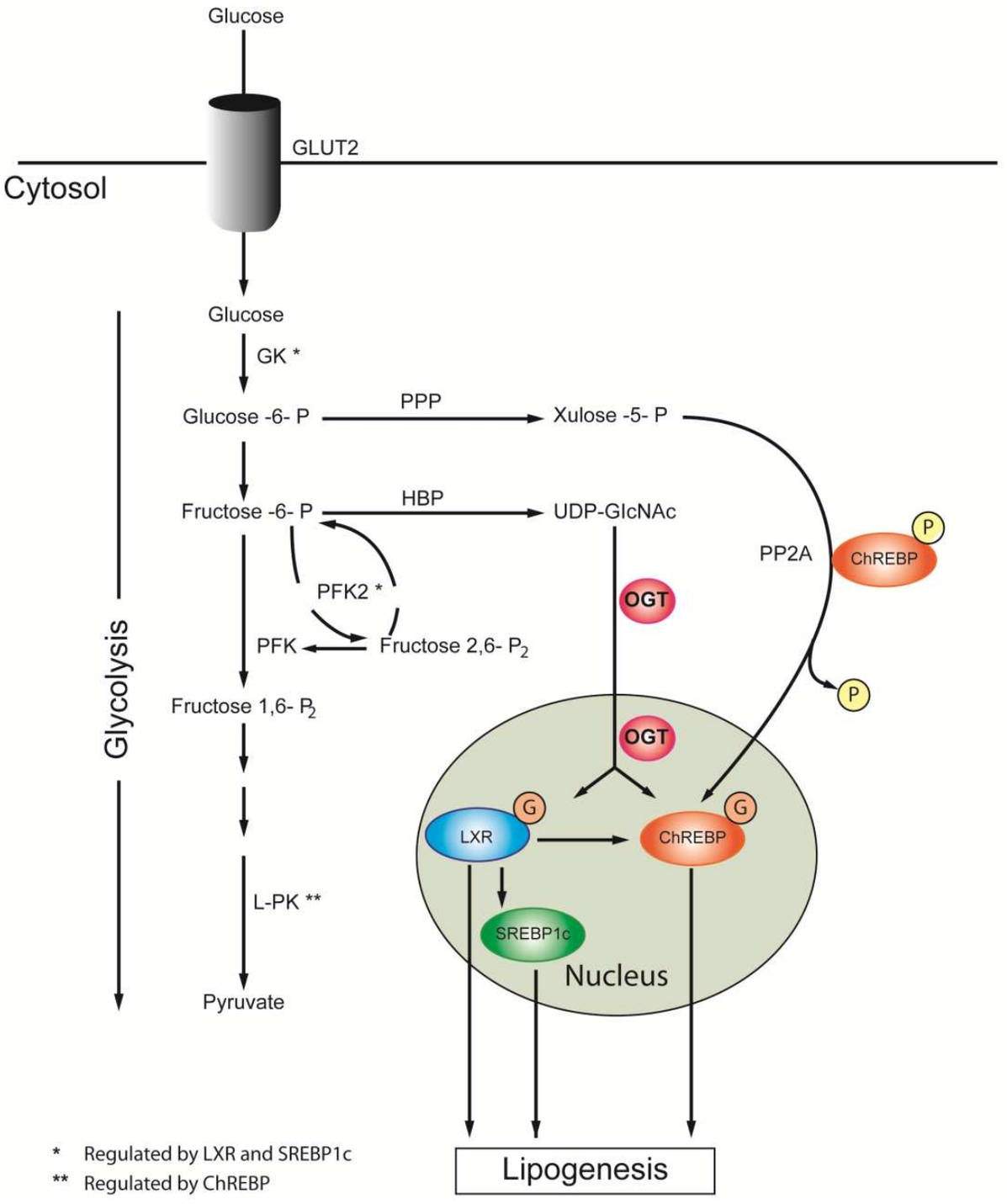

Figure 6. Glucose-mediated regulation of hepatic lipogenesis

5. Cross-talk between O-GlcNAc- and insulin signaling

Studies in *C.elegans* demonstrate that O-GlcNAc cycling phenotypes are very sensitive to insulin as well as nutrient composition and that levels of insulin and nutrients influence the role of O-GlcNAc cycling and vice versa (Mondoux et al., 2011; Hanover et al., 2010; Hanover et al., 2010; Whelan et al., 2008). Intriguingly, O-GlcNAc-marked promoters in *C.elegans* are biased toward genes associated with PIP3 signaling, hexosamine biosynthesis, and lipid/carbohydrate metabolism (Love et al., 2010a). Defects in O-GlcNAc cycling results in deregulation of genes necessary for carbohydrate and lipid metabolism in response to insulin (Forsythe et al., 2006; Hanover et al., 2010) suggesting that both O-GlcNAc cycling and insulin-signaling are required for a robust and adaptable response to hyperglycemia. Several studies have implicated O-GlcNAc cycling in the development of insulin resistance (reviewed in (Mondoux et al., 2011)). Mice overexpressing OGT in muscle or fat and mammalian cells overexpressing OGA develop insulin resistance (McClain, 2002; Arias et al., 2004; Vosseller et al., 2002). Later studies revealed that a subset of OGT was able to transiently translocate to the plasma membrane via association with PIP3 generated by insulin-activated PI3K (Yang et al., 2008). In response to increased glucose metabolism, PIP3-associated OGT can O-GlcNAcylate IR, IRS and Akt antagonizing insulin signaling (Yang et al., 2008; Whelan et al., 2010). Moreover, OGT may also interact with the mTOR pathway (Hanover et al., 2010). As mentioned in section 3.2.3, the downstream target for insulin signaling, FoxO1, is also modified by O-GlcNAc, apparently via OGT recruitment to PGC1α, providing another mechanism for OGT to contribute to insulin resistance, at least for sustained hepatic glucose production in response to hyperglycemia (Housley et al., 2009). Directing OGT to transcriptional targets implies that PGC1α can integrate multiple nutrient signals to regulate gene expression. Whether OGT via PGC1α or other CAs is also recruited to ChREBP- and LXR-regulated promoters is currently not known. OGT is recruited to and O-GlcNAcylate several coregulators and histone modifying enzymes (acetylases/deacetylases, metylases/demetylases) and even histones themselves (Fujiki et al., 2009; Hanover et al., 2012; Fujiki et al., 2011; Sakabe et al., 2010). Depending on the nutritional stimuli, all components of the transciptional machinery from specific transcription factors to coregulators, histones and RNA polymerase II are subject to epigenetic regulation by acetylation, ubiquitinylation, SUMOylation, phosphorylation and/or O-GlcNAcylation (Rosenfeld et al., 2006; Venteclef et al., 2011; Love et al., 2010b; Kato et al., 2011). The fine-tuning of these modifications determines whether a gene is activated or repressed. Furthermore, as the substrate specificity of OGT is believed to be spatio-temporally regulated by transient interactions with large enzyme complexes, its binding to PIP3 may not occur solely at the plasma membrane, as PI3K is also active in the nucleus where it is involved in regulation of protein-chromatin interactions, transcription and mRNA export (Viiri et al., 2012; Kebede et al., 2012; Okada & Ye, 2009). As protein O-GlcNAcylation is rapidly increased at both the plasma membrane and the nucleus in response to serum-stimulation (Carrillo et al., 2011), OGT-binding to nuclear PIP3 may also be instrumental in transcriptional regulation in response to feeding. Interestingly, nonalcoholic fatty liver

disease is often accompanied by hepatic insulin resistance, metabolic syndrome, and diabetes (reviewed in (Scorletti et al., 2011)) and the sensitivity of OGT to glucose increases with decreasing insulin signaling (Mondoux et al., 2011). These findings suggest that elevated O-GlcNAc cycling on key nuclear proteins contributes to the development of hepatic steatosis. This notion is also in line with the above mentioned observation by Guinez et al (Guinez et al., 2011), where overexpression of OGA reduced hepatic steatosis in db/db mice. A complete summary of a putative glucose-insulin cross-talk in regulation of hepatic *de novo* lipogenesis is depicted in Fig. 7.

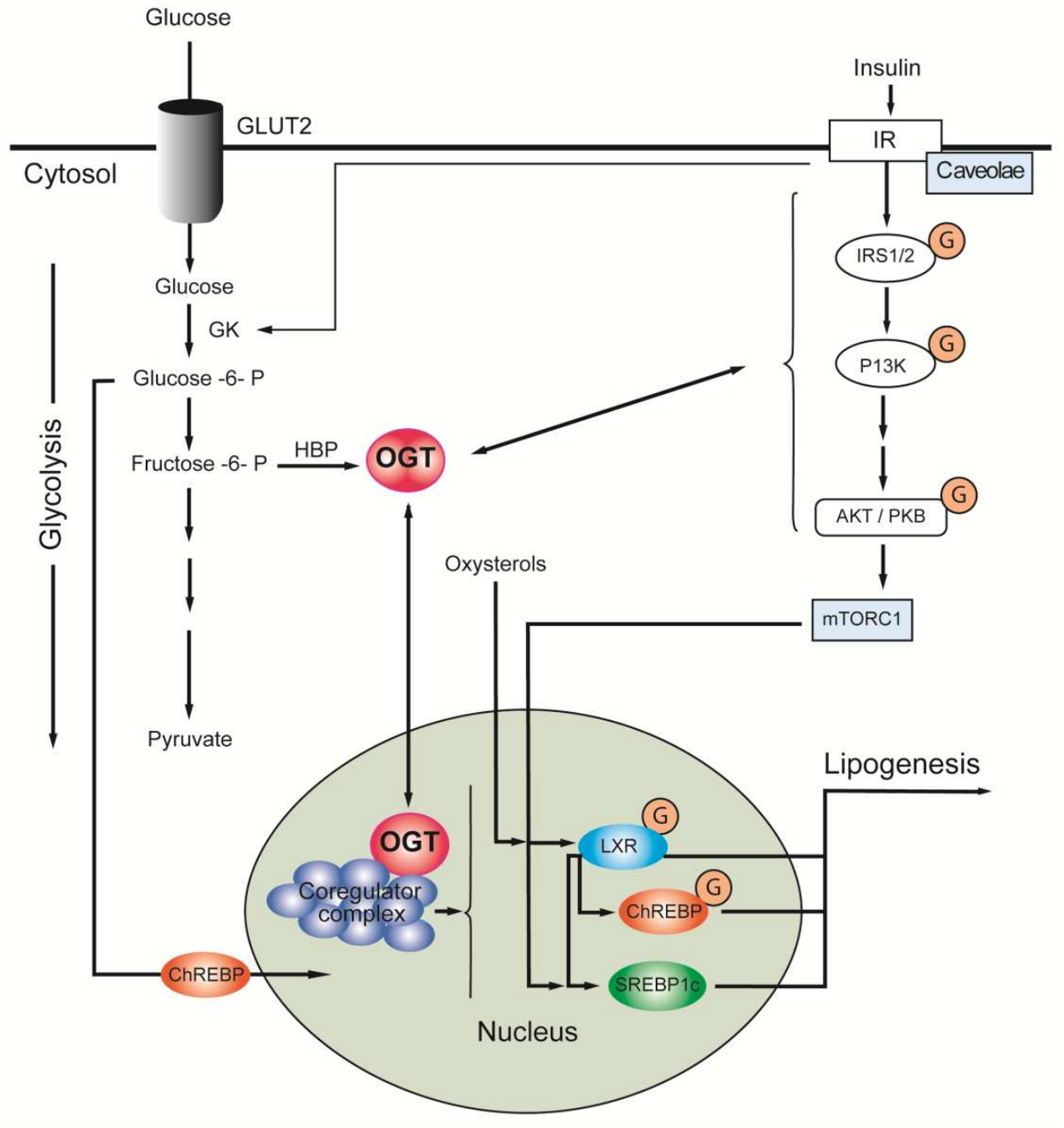

Figure 7. Glucose-insulin cross-talk in regulation of hepatic lipogenesis

6. Concluding remarks

In mice and humans, hepatic *de novo* lipogenesis is activated by a high intake of both glucose and fructose (Scorletti et al., 2011; Schwarz et al., 1995; Schwarz et al., 2003). Fructose increase hepatic hexosamine signaling (Hirahatake et al., 2011) and induce SREBP1c and ChREBP expression in hepatic cells (Matsuzaka et al., 2004; Haas et al., 2012; Koo et al., 2009), which may, in part be mediated by LXR. The response of LXR to glucose has been debated (Lazar & Willson, 2007), but a recent study support the notion of LXR as a glucose/fructose sensor as high sucrose fed mice exhibit elevated hepatic expression of SREBP1c and increased TG levels, which was not observed in LXRα/β double knock out mice (Korach-Andre et al., 2011). LXR increases lipogenesis, in part by activating SREBP1c and ChREBP proteins. Thus, in response to feeding, they can cooperately activate most of the genes required for hepatic lipogenesis and TG secretion. Whether hepatic LXR drives the expression of SREBP1c and/or ChREBP to the same degree under different nutritional conditions is currently not known, as most studies have been performed using synthetic LXR agonists. We have preliminary results showing that hepatic expression of SREBP1c and ChREBP is upregulated in refed control mice and to a lesser extent in STZ-treated hyperglycemic mice, which is not observed in LXRα/β double knock out mice (Bindesbøll et al, unpublished). O-GlcNAc modification of LXR is increased in STZ-treated mice (Anthonisen et al., 2010) and we postulate that O-GlcNAc modification of LXR in response to glucose activates LXR and drives the expression of ChREBP and SREBP1c and in particular the lipogenic genes, ACC and SCD1. Furthermore, RNA Pol II ChIP-Seq data show reduced binding of RNA Pol II to the L-PK promoter and no binding of RNA Pol II to the SCD1 promoter in LXR α/β double knock out mice compared to control mice. Moreover, a novel LXRE immediately downstream of SCD1 was found, to which LXR bound more strongly than the previously published upstream LXR binding site (Boergesen et al., 2012). This suggests an important role for LXR as an upstream activator of ChREBP-mediated transcription and argues for LXR acting independently on the SCD1 promoter, at least under certain nutritional conditions. Previous studies have demonstrated that LXR directly activates key lipogenic genes (Joseph et al., 2002), most notably SCD1 in the liver of SREBP1c knockout mice (Liang et al., 2002; Chu et al., 2006). Why there would be a need for LXR to activate lipogenic genes directly, may be explained by the nutritional conditions and redundancy in the system. Oxysterols bind the endoplasmic reticulum resident Insig protein and could inhibit the proteolytic maturation of SREBP1c (Radhakrishnan et al., 2007). This would limit transcription by SREBP1c, and direct activation by LXR would be required to stimulate lipogenesis. In the absence of active SREBP1c, however, LXR may act in concert with ChREBP in regulating lipogenic expression. A recent study show that hepatic overexpression of ChREBP induces SCD1 expression and hepatic steatosis, but not insulin resistance (Benhamed et al., 2012). Whether overexpression of ChREBP affected LXR protein expression and transactivation of the SCD1 promoter was not investigated in this study. In later studies, it would be interesting to investigate the SCD1 expression and activity in livers or hepatocytes with targeted deletion of ChREBP. Benhamed et al (Benhamed et al., 2012) also showed that ChREBP expression was increased in liver biopsies from patients with

steatosis and decreased in liver of patients with severe insulin resistance, suggesting that ChREBP, alone or in combination with LXR, drives SCD1 expression and steatosis independent of insulin resistance. This is in line with recent human studies showing no relationship between hepatic TG accumulation and insulin resistance (Cohen et al., 2011; Hooper et al., 2011). Thus, hepatic steatosis can either be the result or cause of hepatic insulin resistance. The mechanisms of hepatic insulin resistance is still not clear (Farese, Jr. et al., 2012), but may involve specific lipids, nutrition-induced metabolites and PTMs including O-GlcNAc. Hepatic TG synthesis may be a protective mechanism to limit accumulation of toxic free fatty acids, liver damage and fibrosis (Choi & Diehl, 2008) where particularly SCD1 seem to play a protective role (Li et al., 2009).

As LXR is shown also to act anti-inflammatory in liver (Wouters et al., 2008; Venteclef et al., 2010), LXR activation may be an important compensative mechanism in response to excess nutrients to limit liver damage, inflammation and fibrosis. SUMOylation is an important ligand-activated transrepressional PTM of LXR on inflammatory genes (Venteclef et al., 2011) and future studies in our laboratory aim to elucidate a putative cross-talk between OGT and E3 ligases (SUMO conjugating enzymes) in liver in response to excess nutrients, especially high sugar levels (glucose and fructose). The relative roles of LXR, SREBP1c and ChREBP in driving lipogenesis is clearly dependent on both insulin and glucose signaling and cross-talk between these pathways. Both phosphorylation and GlcNAcylation appear instrumental in hepatic lipogenesis and future focus in our laboratory will be to elucidate a possible cross-talk between these PTMs, endogenous LXR ligands and interacting CAs in response to various feeding conditions (high glucose, fructose and/or fatty acids, cholesterol) and the impact on downstream ChREBP, SREBP1c and lipogenic enzyme expression and activity. ChIP and reChIP analysis in combination with loss of function studies have become powerful tools to analyze activation of specific genes by specific transcription factors in response to extracellular stimuli. By these methods, we anticipate that the signaling mechanisms and relative roles of LXR, ChREBP, SREBP1c and cooperating transcription factors in driving hepatic *de novo* lipogenesis will be revealed in the not too distant future.

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