Molecular Mechanisms of Insulin Resistance in Chronic Liver Disease

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Abbreviations

ACC, acetyl-CoA carboxylase; *AMPK*, AMP activated protein kinase; *ALT*, alanine aminotransferase; *AP*, alkaline phosphatase; *AST*, aspartate aminotransferase; *AS160*, Akt substrate of 160 kDa; *A1AT*, α1-antitrypsin; *BMI*, body mass index; *CaMKK*, calmodulin-dependent protein kinase kinase; *CAR*; constitutive active/androstane receptor; *c-DNA*, complementary DNA; *CK-18*, cytokeratin-18; *CREB*, cyclic AMP response element binding protein; *CT*, threshold cycle; *CTMP*, carboxy-terminal modulator protein; *DMEM*, Dulbecco's modified Eagle's medium; *ELISA*, enzyme-linked immunosorbent assay; *ER*, endoplasmatic reticulum; *FFA*, free fatty acids; *Foxo1 and 3*, forkhead transcription factors; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *GGT*, γ-glutamyl transpeptidase; *GLUT*, glucose transporter; *GSK-3β*, glycogen synthase kinase 3β; *GTT*, glucose tolerance test; *G6Pase*, glucose-6-phophatase; *HBV*, hepatitis B virus; *HCC*, hepatocellular carcinoma; *HCV*, hepatitis C virus; *HFD*, high fat diet; *HNF4*, hepatocyte nuclear factor 4; *HOMA-IR*, homeostasis model assessment of insulin resistance; *Hsp90*, heat shock protein 90; *IDF*, International Diabetes Federation; *IFNα*, Interferon α; *IL-6*, interleukin-6; *INR*, international normalized ratio; *IRS 1-4*, insulin receptor substrates 1-4; *ITT*, insulin tolerance test; *JNK*, jun-N-terminal kinase; *LXR*, liver X receptor; *MAPK*, mitogen activated protein kinase; *MEK*, MAPK kinase; *mRNA*, messenger ribonucleic acid; *mTOR*, mammalian target of rapamycin; *MTP*, microsomal triglyceride transfer protein; *NAFLD*, non alcoholic fatty liver disease; *NAS*, NAFLD activity score; NASH, non alcoholic steatohepatitis; *OA*, okadaic acid; *ORF*, open reading frame; *PDK1*, phosphoinositide-dependent kinase 1; *PEPCK*, phosphoenolpyruvatcarboxykinase; *PGC1α*, peroxisome proliferator–activated receptor 1α; *PHLPPα*, PH domain leucine-rich repeat protein phosphatase *α*; *PIAS1*, protein inhibitor of activated STAT 1; *PIP_3*, phosphoinositol-3-phosphates; *PI3K*, phosphatidyl-inositol-3-kinase; *PKB/Akt*, protein kinase B; *PKC*, protein kinase C; *PP2Ac*, protein phosphatase 2A catalytic subunit; *PRMT1*, protein arginine methyl transferase 1; *qRT-PCR*, quantitative (real time) reverse transcription polymerase chain reaction; *PTEN*, phosphatase and tensin homolog; *SEM*, standard error of the mean; *SHIP*, src-homology 2-containing inositol 5' phosphatase; *SHP*, small heterodimer partner; *shRNA*, short hairpin RNA; *SH2*, scr-homology-2; *SOCS*, suppressor of cytokine signaling 3; *SREBP1c*, sterol regulatory element binding protein 1c; *STAT*, signal transducer and activator of transcription; *SVR*, sustained virologic response; *TG*, triglycerides; *tg*, transgenic; *TMAO*, trimethylamine N-oxide dihydrate; TNFα, tumor necrosis factor α; *TORC2*, mTOR complex 2; *TORC2*, transducer of regulated CREB activity 2; *TUDCA*, tauroursodeoxycholic acid; *T2DM*, type 2 diabetes mellitus; UDCA, ursodeoxycholic acid; *UPR*, unfolded protein response; *VLDL*, very low density lipoprotein; *4-PBA*, 4-phenyl butyric acid;

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1. Introduction

1. Hepatitis C Virus Infection

Epidemiology and natural history of hepatitis C

Hepatitis C Virus (HCV) infection is one of the major causes of chronic liver disease. According to recent WHO estimates, the prevalence of HCV is approximately 2.2% - affecting approximately 130 million people worldwide (1). There are different genotypes of the HCV virus. The most common genotypes are genotype 1, 2, 3 and 4.
Severity of chronic hepatitis C is assessed by histological analysis of inflammation and fibrosis in a liver biopsy sample, according to a scoring system. The most widely used score is the Metavir score (2). Progression of chronic hepatitis C varies between individuals. Up to 85% of patients infected with HCV develop chronic liver disease and are at risk for fibrosis progression to cirrhosis of the liver, which develops in 20-30% of cases (3).
Cirrhosis in HCV infection is a common cause of hepatocellular carcinoma and end-stage liver disease, necessitating liver transplantation. Risk factors for a progressive course of chronic hepatitis C are age, male gender, alcohol consumption, diabetes and steatosis. Interestingly, when insulin resistance, an earlier and more sensitive parameter of glucose metabolism derangement, is added to logistic regression, the association between steatosis and fibrosis disappears (4).
Until present there is no protective vaccine available. The current standard treatment is Peginterferonα combined with Ribavirin. It has to be given for 6 to 12 months dependent on the HCV genotype (5). Still, only 50% of patients reach a so-called "sustained virologic response (SVR)", determined when there is no detectable HCV-RNA in the blood 6 months after the end of antiviral therapy, and can be considered as cured from HCV infection. Response to the treatment is monitored by HCV-RNA in the blood at weeks 4 and 12 of treatment and undetectable HCV-RNA is a good predictor of response (6). Response to therapy is dependent on HCV genotype and viral load. Further host factors such as age, race, body mass index (BMI), insulin resistance, hepatic steatosis and fibrosis might play a role (7, 8).

The Hepatitis C Virus

The Hepatitis C Virus (HCV) has been discovered in 1989 (9). It is the sole member of the genus hepacivirus within the Flaviviridae family, an enveloped single stranded sense RNA virus. The positive stranded RNA genome comprises 9600 nucleotides. It contains a 5' non coding region, a single long open reading frame (ORF) encoding a polyprotein precursor of approximately 3000 amino acids and a 3' non coding region. The polyprotein precursor is posttranslationally processed by cellular and viral proteases into structural and non-structural proteins. The structural proteins include the core protein that forms the viral nucleocapsid and the envelope proteins E1 and E2. The nonstructural proteins include the NS2-NS3 autoprotease, the NS3 serine protease (a RNA helicase), the NS4A cofactor for NS3, the NS4B involved in formation of intracellular membrane particles and the NS5A and NS5B RNA-dependent RNA polymerase (10, 11).

Insulin Resistance in chronic hepatitis C

Over the past 10 years many epidemiological studies have pointed out the role of insulin resistance as an important host factor in chronic hepatitis C. Insulin resistance is defined as a condition where higher insulin concentrations are needed to achieve normal glucose metabolism or normal insulin concentrations fail to achieve normal glucose metabolism (12). It is the major pathogenic factor of type 2 diabetes mellitus (T2DM) and the metabolic syndrome. A well-accepted method to measure systemic insulin resistance in patients is by the HOMA-IR (Homeostasis model assessment of insulin resistance). It is calculated by the following formula: fasting glucose (mmol/l) x fasting insulin (mIU/l) /22.5 (13). Hepatic insulin resistance has to be distinguished from systemic insulin resistance as it affects only the liver and cannot be measured by the HOMA-IR.

In chronic hepatitis C, prevalence of insulin resistance and the risk to develop T2DM are clearly increased compared to healthy subjects or subjects with chronic liver disease other than HCV, e.g. Hepatitis B Virus (HBV) infection (7, 14-19).

A relationship between insulin resistance and the severity of chronic hepatitis C has been described. Several studies have identified insulin resistance and diabetes as independent predictors of fibrosis progression and development of cirrhosis (4, 20-24). Also resistance to antiviral therapy has been assigned to insulin resistance in clinical trials (21, 25, 26) and improvement of insulin resistance during therapy (27, 28) in SVR in response to antiviral treatment has been observed (25).

Hepatic steatosis is the histopathological term for accumulation of triglycerides in hepatocytes and has also been associated to HCV infection (19). Experimental and clinical data have shown that HCV genotype 3a can directly induce lipid accumulation in hepatocytes by interference with VLDL (very low density lipoprotein) assembly and upregulation of sterol regulatory element binding protein 1c (SREBP1c) (29-33). In genotypes other than 3a, steatosis is a consequence of insulin resistance development and is therefore also related to fibrosis progression and resistance to antiviral therapy (7, 34). Metabolic aspects of chronic HCV infection therefore are to some extend similar to non alcoholic fatty liver disease (NAFLD).

The relationship between insulin resistance and HCC remains to be further elucidated. A study of NAFLD propose a role for insulin resistance in carcinogenesis (35).

Hence, insulin resistance and steatosis of the liver in a setting of chronic hepatitis C have clinical and prognostic implications (Figure 1). But pathogenesis of insulin resistance in HCV is still incompletely understood (see 1.6.) and its understanding would help to improve treatment and prevent complications.

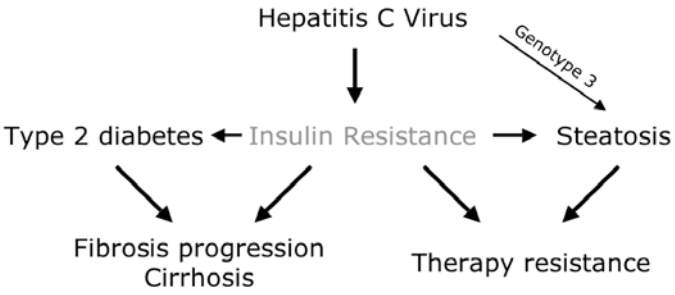

Figure 1: *Insulin Resistance in chronic hepatitis C*

2. Non alcoholic fatty liver disease (NAFLD)

Non alcoholic fatty liver disease (NAFLD) refers to the presence of hepatic steatosis that is not associated with significant consumption of alcohol or other liver disease. It is considered to be the liver manifestation of the metabolic syndrome, a prosperity disease with increasing prevalence. Presumably NAFLD is the most common chronic liver disease in the western world. The precise prevalence of NAFLD is unknown. Data from different studies varies according to the method used to detect steatosis and the population studied. A recent representative study estimated the prevalence of NAFLD and NASH in the general population at up to 33% and 17%, respectively, and 80% and 40%, respectively, in patients with metabolic syndrome (36-38).
Simple fatty liver might be a benign disease. However in its progressive form "non alcoholic steatohepatitis" (NASH) up to 20% of patients will develop cirrhosis, and some of them will progress to liver failure (9%) or hepatocellular carcinoma (HCC) (1%) (39).
Unfortunately there are currently no pharmacological therapy options for the prevention of NAFLD or progression of the disease. Standard of care are lifestyle modifications with weight loss by increase in physical activity and change in dietary habits. Insulin sensitizing and hepatoprotective drugs have been tested. So far no agent has been demonstrated to prevent disease progression (40, 41).
Another difficulty in disease management is the inability to predict which patient will develop liver-related morbidity. Non-invasive markers predicting NASH in NAFLD patients are needed to identify patients at risk. In vivo assessment of cytokeratin-18 fragments in the blood has recently been proposed as a potential non-invasive marker for NASH (42, 43). Cytokeratin-18 is the major intermediate filament in the liver and is cleaved by caspases when hepatocytes undergo apoptosis (44).

3. Overexpression of PP2Ac in chronic hepatitis C

Interferonα (IFNα) signaling is inhibited in chronic hepatitis C

Interferonα (IFNα) signaling is an important pathway activated in spontaneous response to HCV infection and in standard antiviral therapy. Resistance to IFNα signaling resulting in chronic HCV infection and non-response to treatment is still incompletely understood. Studies from our laboratory revealed that the expression of viral proteins in cells in culture and in B6HCV transgenic mice inhibits Jak-STAT signaling (45, 46).

Protein Phosphatase 2A (PP2A)

Phosphorylation and dephosphorylation of proteins are essential post-translational modifications in the regulation of signal transduction. Protein phosphatases therefore play an important role in regulating protein kinase cascades and the cellular localization of various proteins, e.g. transcription factors. There are several serine/threonine phosphatases described: PP1, PP2A, PP2B (calcineurin), PP2C, PP3, PP4 and PP5.
PP2A is ubiquitously expressed in eukaryotic cells and represents approximately 1% of total cellular proteins (47). Deletion of PP2A catalytic subunit in mice is lethal supporting its

essential role (48). PP2A is a multimeric protein consisting of 3 subunits: a catalytic 36 kDa C subunit and a scaffolding 65 kDa A subunit (or PR65), which form a constitutive complex. Two isoforms of the C subunit and of the A subunit exist (α and β). This A-C core dimer can interact with any of the various regulatory B subunits from three different families: B, B' and B'' (or PR55, PR61 and PR72). Assembly of distinct holoenzymes plays a role in directing the phosphatase to distinct substrates and to distinct cellular compartments (49).
More than 30 protein kinase activities are influenced by PP2A in vitro and several kinases form complexes with PP2A (49). These kinases are involved in diverse cellular processes: metabolism, DNA replication, transcription, translation, cell-cycle progression, morphogenesis, development, transformation and stress response (50). Among the PP2A substrates there are PKB/Akt, PKC, p70S6Kinase, AMP Kinase, MEK, ERK, JNK (49, 51).
The regulation of PP2A is tightly controlled (52). However, downregulation of PP2A by all-trans-retinoic acid (53) or through peroxisome proliferator activated receptor γ (54) and upregulation through colony stimulating factor 1 have been reported (55).
Okadaic acid (OA) is a natural inhibitor of PP2A. In vitro it inhibits PP2A with an IC_{50} of 0.1nM. Below concentrations of 1 μM, OA has been considered to be selective for PP2A (56).

Upregulation of PP2A inhibits IFNα signaling and increases viral replication

Upregulation of Protein Phosphatase 2A (PP2A) was observed in HCV protein expressing cells, in the liver of B6HCV transgenic (tg) mice and in human liver biopsies from patients infected with HCV when compared to healthy controls (57). It has been shown that overexpression of PP2A inhibited protein arginine methyl transferase 1 (PRMT1) enzymatic activity and therefore lead to hypomethylation of STAT1. Hypomethylation of STAT1 facilitates binding to its inhibitor protein inhibitor of activated STAT1 (PIAS1) that leads to decreased STAT1 binding to target genes in response to IFNα (57). Inhibition of PRMT1 by PP2A additionally leads to hypomethylation of viral helicase NS3 that increases NS3 unwinding activity and therefore increases viral replication (58). Treatment with S-Adenosyl-L-methionine and Betaine has been shown to restore STAT1 methylation, improve IFNα signaling, and antiviral response in hepatocytes in culture (59). In a clinical trial in the Department of Gastroenterology and Hepatology at the University Hospital in Basel we are currently testing whether addition of S-Adenosyl-L-methionine and Betaine to standard antiviral therapy can overcome resistance to antiviral therapy in HCV patients that did not respond to IFNα therapy, so called non-responders.
Interestingly, PP2A is also overexpressed in HBV infection, a virus of the hepadnaviridae family of hepatotropic DNA viruses. In HBV expressing hepatoma cells and in liver biopsies of HBV infected patients we found an overexpression of PP2A and an inhibition of IFNα signaling (60).

Viral induced ER stress response leads to the upregulation of PP2A

The Endoplasmatic reticulum is a membranous network responsible for protein synthesis, protein folding and export to the Golgi complex. Folding is processed by foldases and facilitated by molecular chaperones that shield unfolded regions from surrounding proteins. Only correctly folded proteins are exported. During pathological stress situations such as viral infection but also glucose deprivation or lipid accumulation, the protein folding

machinery is overload with unfolded proteins and induces an unfolded protein response (UPR) or ER stress response (61, 62). The ER stress response induces an increase in folding capacity of the ER through transcriptional upregulation of genes, e.g. chaperones (63). Also chemical chaperones such as 4-phenyl butyric acid (4-PBA), trimethylamine N-oxide dihydrate (TMAO) and dimethyl sulfoxide can increase folding capacity of the ER (64).
Recently it has been shown, that the ER stress response can induce insulin resistance in mice (65, 66).
The fact that a RNA- and a DNA-virus both upregulate PP2A lead us to the study of virus induced endoplasmatic reticulum stress (ER stress). It is well known that viral infection can induce ER stress and indeed, it has been published that the expression of HCV structural proteins E1, E2 and HCV core induce an ER stress response (67-69).
It has been shown in our laboratory that HBV and HCV are both able to induce an ER stress response. During ER stress a calcium release from the ER to the cytoplasm activates cyclic AMP response element binding protein (CREB). Activated CREB then binds to its binding site on the PP2A promoter region and upregulates its transcription (70).

4. Regulation of glucose homeostasis in the liver

The liver is the central organ in energy metabolism, and insulin is the primary anabolic hormone promoting energy storage in the fed state (71). High portal insulin concentrations rapidly trigger stimulation of glycogensynthesis, lipogenesis, inhibition of VLDL secretion and suppression of gluconeogenesis and glycogenolysis. These processes are regulated by phosphorylation and dephosphorylation of metabolic pathways, regulation of gene expression and mRNA stability (72, 73). Storage of glucose as glycogen and suppression of gluconeogenesis are regulated by insulin, glucose and the parasympathetic nerve (73). After an over night fast, up to 60% of circulating glucose are derived from hepatic glycogenolysis and at least 40% from gluconeogenesis. After prolonged fasting (24 to 36 hours) hepatic gluconeogenesis primarily from alanine is the sole source of blood glucose (74, 75).
In peripheral tissues as muscle and adipose tissue, in the fed state insulin increases glucose uptake by translocation of the GLUT4 transporter to the cell surface (76). Up to 75% of insulin dependent glucose disposal occurs in skeletal muscle.

Insulin signaling

The insulin receptor is ubiquitously expressed and is a member of the receptor tyrosine kinases. Insulin binding to the insulin receptor α-subunit leads to a conformational change in the intracellular β-subunit and to derepression of its tyrosine kinase domain resulting in transphosphorylation of the β-subunit. When phosphorylated, tyrosine kinase domains have enhanced activity towards non-receptor substrates (77). There are different non-receptor substrates: insulin receptor substrates 1-4 (IRS-1, IRS-2, IRS-3, IRS-4), which are the most specific substrates, but also Gab-1, $p60^{dok}$, Cbl, SHc and APS (78). Among these, IRS-1 and IRS-2 are best described and are expressed in many tissues, predominantly in the liver. IRS-1 and IRS-2 knockout mice have an insulin resistant phenotype (79).
The phosphorylated tyrosines in these substrates act as "docking sites" for scr-homology-2 (SH2) domain containing proteins. Many of these proteins are adaptor proteins, the most important SH2 adaptor proteins are p85 and Grb-2 (79). Interaction of IRS to p85-PI3K leads to activation of PI3K p110 catalytic subunit, a central kinase in mediation of metabolic actions of insulin. PI3K activity can be inhibited by Wortmannin. It catalyses the phosphorylation of phosphoinositides on the 3-position generating phosphoinositol-3-phosphates (PIP_3) (80). Important inhibitors of this process are PTEN and SHIP.
PIP_3 regulate the AGC family of serine/threonine kinases, among them phosphoinositide-dependent kinase 1 (PDK1) (81). Generation of PIP3 also leads to translocation of Protein kinase B (PKB/Akt) to the plasma membrane (82). PDK1 phosphorylates PKB/Akt on Thr308 in the kinase domain (81). Full activation of PKB/Akt further requires phosphorylation on Ser473 on its hydrophobic motif by mTOR complex 2 (TORC2) (83) leading to stabilization of the active conformational state (82, 84). PKB/Akt is a serine/threonine kinase that has a central role in transmission of insulin signal by phosphorylation of glycogen synthase kinase 3 (GSK-3), Forkhead transcription factors (FoxO1 and 3) and cAMP response element-binding protein (CREB) (79, 85). PKB/Akt can be negatively regulated by protein phosphatase 2A (PP2A) and PH domain leucine-rich repeat protein phosphatase (PHLPPα) by directly dephosphorylating S473 and/or Thr308 (86, 87). Carboxy terminal modulator protein (CTMP) is another negative regulator that reduces PKB/Akt activity by binding to its carboxy-terminal domain and preventing its phosphorylation (88).
Another downstream target of Akt is mTOR, an important kinase regulating protein synthesis, cell growth and metabolism (89). mTOR activation leads to a negative feedback loop to insulin signaling inducing phosphorylation of IRS-1 on Ser307 by S6Kinase, inhibiting its tyrosine phosphorylation (90).
PKB/Akt phosphorylates and inactivates GSK-3 hereby inducing activation of glycogen synthase in order to increase glycogen synthesis (91). One the other hand, by phosphorylation induced nuclear exclusion of Foxo1, PKB/Akt inhibits expression of key enzymes of gluconeogenesis: phosphoenolpyruvatcarboxykinase (PEPCK) and glucose-6-phophatase (G6Pase) (92, 93). Akt induced inhibition of gluconeogenesis involves Foxo1 interaction with peroxisome proliferator–activated receptor 1α (PGC1α) (94-96). It has been reported recently, that PKB/Akt might directly inhibit PGC1α by phosphorylation and prevention of promoter binding (97).
In the liver insulin signaling thereby is a crucial pathway for the regulation of glycogensynthesis and glycogenolysis, gluconeogenesis but also lipogenesis (79).

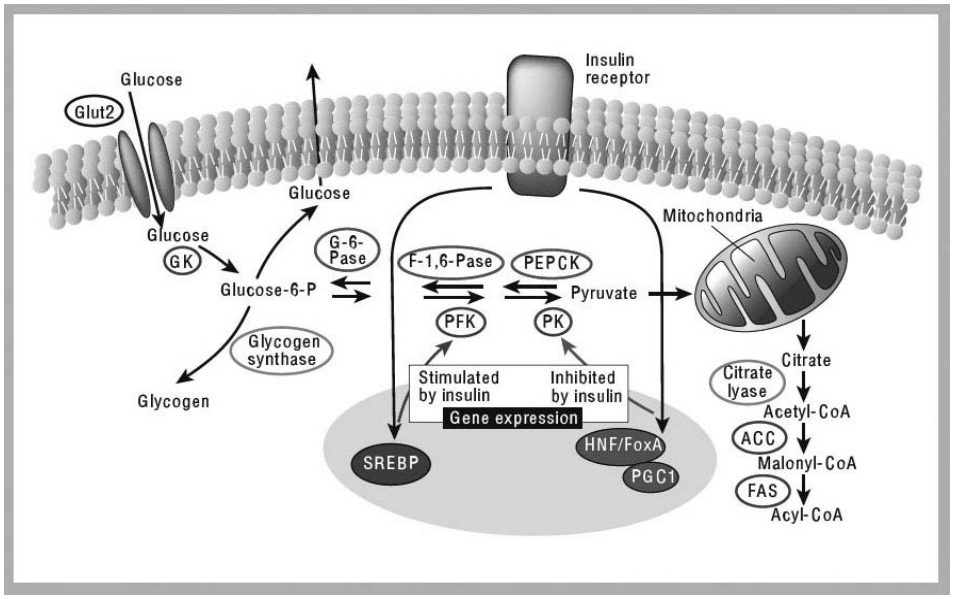

Figure 2: *Regulation of glucose metabolism in the liver (79)*
In hepatocytes insulin stimulates the utilization and storage of glucose as glycogen and lipid by stimulating glycogensynthase and citrate lyase and upregulation of SREBP, ACC and FAS. It further inhibits the production of glucose from aminoacids by inhibition of Forkhead transcription factors, PGC1α and HNF4.

AMP activated kinase (AMPK)

AMP activated kinase (AMPK) is an energy sensor that regulates cellular metabolism in response to nutrient deficiency. When AMP levels are high, AMPK is activated leading to stimulation of energy producing processes such as glucose uptake or lipid oxidation and to inhibition of energy consuming pathways such as gluconeogenesis or lipogenesis. It controls whole body energy homeostasis by the regulation of glucose- and lipid-metabolism in multiple tissues (98).
AMPK is a heterotrimeric protein consisting of a catalytic α and regulatory β and γ subunits. Upon AMP binding to the γ subunit, AMPK is allosterically activated and phosphorylated on Thr172 by the upstream kinase LKB1 (99) or through rise in intracellular Ca^{2+} ions by CaMKK (100). In the liver, activation of AMPK leads to suppression of gluconeogenesis regulated by PEPCK and G6Pase. Gluconeogenesis is insufficiently suppressed in liver specific AMPKα2 knockout mice (101).
Metformin is a drug widely used as a treatment for type 2 diabetes mellitus since 1979. It inhibits hepatic gluconeogenesis and therefore improves blood glucose levels. Recently, it has been shown that metformin induces phosphorylation on Thr172 and activation of AMPK. In this model, AMPK phosphorylates TORC2 leading to its nuclear exclusion and thereby prevents transcriptional induction of gluconeogenic key enzymes by PGC1α coactivation (102). A second model for metformin induced suppression of gluconeogenesis hints at involvement of AMPK induced upregulation of small heterodimer partner (SHP) (103).
There is evidence that PP2A regulates AMPK. Indeed, inhibition of PP2A by Okadaic acid lead to AMPK phosphorylation in rat hepatocytes (104). PP2A was described to inactivate AMPK in pancreatic β-cells (105) and inhibit AMPK phosphorylation in vascular endothelial cells (106).

Liver X receptor (LXR)

Nuclear receptors are ligand activated transcription factors that regulate gene expression in response to hormonal and environmental factors. Liver X receptors (LXRα and LXRβ, also known as NR1H3 and NR1H2) are nuclear receptors that play an important role in the transcriptional regulation of cholesterol- and fatty acid- but also glucose metabolism. Furthermore they have been implicated in the control of immune response and inflammation and might therefore integrate metabolic and inflammatory signaling pathways (107).
Oxidized metabolites of cholesterol – so called oxysterols – are the natural ligands of LXR (108). There are two LXR isoforms: LXRα is highly expressed in the liver and at lower levels in intestinal, adrenal glands, adipose tissue, macrophages and kidney whereas LXRβ is ubiquitously expressed (109).
Recently it has been published that glucose and glucose-6-phosphate can directly bind to and activate LXR (110). Though it is not completely understood how glucose, a hydrophilic ligand, can bind to the hydrophobic ligand binding pocket of LXR (111), this finding shows that not only insulin but also glucose itself has an effect on hepatic glucose homeostasis regulation.
Upon ligand binding, LXR forms heterodimers with the retinoid X receptor (RXR), recruites coactivators or corepressors in order to regulate target gene expression (112). It has been shown, that treatment with LXR agonists improves glucose tolerance in diabetic mice and rats (113, 114). This effect was at least partially due to decreased hepatic gluconeogenesis by inhibition of the gluconeogenic key enzymes PEPCK and G6Pase. Interestingly glucose, similar to oxysterol LXR ligands, inhibits expression of PGC1α, PEPCK and G6Pase in HepG2 cells grown in low glucose media (110).
LXR as well as other nuclear receptors have recently been found to be phosphorylated proteins (115). It was shown that LXRα is phosphorylated on Serine 198. There is growing evidence that phosphorylation of LXR might regulate LXR induced gene expression (116, 117). The role of PP2A as a serine/threonine kinase in regulating LXR has to be elucidated.
Another hepatic nuclear receptor involved in regulation of gluconeogenesis is constitutive active/androstane receptor (CAR) (118). It has been described that activation of CAR by phenobarbital leads to a complex formation of the CAR-Hsp90 complex with PP2A (119). Its translocation to the nucleus as the initial step for activation by phenobarbital is regulated by dephosphorylation at Ser202a (120). Whether CAR is dephosphorylated on serine 202 by PP2A is discussed but needs to be proven.

5. *Insulin resistance in the pathophysiology of chronic liver disease*

Insulin resistance development by hepatitis C virus

Unless insulin resistance development in chronic hepatitis C is well documented, it is not yet completely understood how the HCV virus interferes with metabolic signaling pathways.
Different models of HCV induced induction of steatosis and insulin resistance come from HCV transgenic mouse models.
One of the HCV core transgenic (tg) mice, that expresses the core of HCV genotype 1b under the control of HBV regulatory elements (31) developed an insulin resistant state at the age of 2 months and steatosis of the liver at the age of ≥ 3 months (121). When fed with a high fat diet, HCV core tg mice developed diabetes due to TNFα induced impaired IRS1-Y phosphorylation (121). A mechanism dependent on the proteasome activator PA28γ for HCV core induced insulin resistance development through degradation of IRS2 and hypophosphorylation of IRS-1 and PKB/Akt has been proposed (122). Moreover it has been shown that HCV core protein interferes with the activity of the hepatic microsomal triglyceride transfer protein (MTP) thereby inhibiting very low density lipoprotein (VLDL) assembly and inducing hepatic steatosis (32).
Similarly, HCV tg mice that express either the entire HCV polyprotein or the HCV structural proteins of genotype 1b under the control of an albumin promoter develop hepatic steatosis at the age of ≥ 10 months (123).
Finally, a third model of HCV tg mice, the B6HCV mice, express the entire HCV ORF genome of genotype 1b under the control of a hepatocyte specific α1-antitrypsin promoter (A1AT) (46, 124). Steatosis and inflammatory infiltrates were detected at an age of 3 to 18 months (46, 124). As mentioned, we observed overexpression of PP2A in the liver of these mice (57).
It has been observed that IRS-1 and -2 protein levels were downregulated in HCV patient liver biopsies with fibrosis progression, in the liver of HCV core tg mice and HCV core expressing HepG2 cells (125). A cell culture model has been developed where Suppressor of cytokine signaling 3 (SOCS3) upregulation leading to degradation of IRS-1 and -2 would inhibit insulin signaling at the levels of of PI3K and PKB/Akt phophorylation (125). In HCV patients with sustained virologic response (SVR) after antiviral treatment, IRS-1 and -2 expression increased consistent with lower HOMA (126).
Decreased IRS-1 expression has also been observed in HCV core expressing Huh7 cells of both HCV genotype 3a and 1b (127). Unless different clinical studies have found an upregulation of SOCS3 in patients with HCV and insulin resistance (128, 129), SOCS3 expression was normal in these cell culture systems. HCV core induced IRS-1 degradation has been found to be genotype specific, involving SOCS7 in genotype 3a but mTOR in genotype 1b (127).
Further insulin signaling has been studied in 42 biopsies from non-obese and non-diabetic HCV patients and 10 non-healthy controls. In this study, insulin receptor and IRS-1 expression was intact but tyrosine phosphorylation of IRS-1 and its association with PI3K, and PKB/Akt phosphorylation were inhibited (130).
It is further interesting that PP2A, that is upregulated by a HCV induced ER stress response, interferes with insulin signaling in adipocytes. Indeed in 3T3-L1 cells, PP2A negatively regulates the insulin signaling cascade at the level of PKB/Akt and GSK3β phosphorylation whereas IRS-1 expression and IRS-1 tyrosine phosphorylation were not affected (131).

Insulin resistance as a key factor in pathogenesis of NAFLD

Little is known about the pathophysiology and natural history of NAFLD and NASH. A so called “two hit hypothesis” has been proposed in 1998 (132). In this model insulin resistance plays a key role in the initiation of the disease or “first hit”. The second hit is still controversially discussed (36). Oxidative stress and cytokine mediated liver-injury might play a role (133).

Hypothetical models support subsequent development of peripheral and later hepatic insulin resistance. Peripheral insulin resistance increases flux of free fatty acids (FFA) to the liver by increased β-oxidation in the adipose tissue whereas hepatic insulin resistance promotes increased de novo lipogenesis both leading to hepatic triglyceride accumulation (134).

In addition to these two mechanisms, lipid accumulation in hepatocytes can theoretically result from impairment in oxidation of FFA at the mitochondria or export of TG in form of VLDL particles (135).

Indeed, obesity might lead to peripheral insulin resistance and secretion of proinflammatory cytokines from the adipose tissue inducing peripheral and also hepatic insulin resistance. It has been shown that chronic exposure to IL-6 leads to hepatic insulin resistance in mice (136). Also chronic exposure to TNFα has been shown to inhibit insulin signaling in HepG2 (137, 138).

Interestingly there is a role for ER stress in insulin resistance development. Leptin deficient ob/ob mice and mice on high fat diet revealed obesity induced ER stress response, leading to hyperactivation of Jun N-terminal Kinase (JNK) and inhibition of insulin signaling by serine phoshorylation of IRS-1 (65). It was shown that 4-phenyl butyric acid, a chemical chaperone, alleviates ER stress and restores hepatic insulin signaling and systemic insulin sensitivity in ob/ob mice (66).

2. Aims

1. Insulin Resistance in the pathophysiology of chronic hepatitisC

The aim of the project was to study the pathophysiology of insulin resistance in chronic hepatitis C.

We hereby studied the role of hepatic Protein Phosphatase 2 A (PP2A) overexpression and its interference with

- Insulin signaling,
- AMPK signaling and
- Glucose induced regulation of gluconeogenesis.

Based on the concept that insulin resistance is involved in disease progression and therapy resistance, a better understanding of these mechanisms is required for future development of more effective antiviral drugs to cure HCV infection or slow down fibrosis progression.

2. Insulin Resistance in the pathophysiology of NAFLD

The aim of the second project was to characterize a cohort of patients with non alcoholic fatty liver disease (NAFLD).

We investigated the discrepancy between NAFLD patients and patients with advanced stage of NAFLD: non alcoholic steatohepatitis (NASH) concerning

- Clinical and laboratory tests and
- Cytokeratin-18 fragmentation in the serum.

3. Material and Methods

Material and methods of the experiments shown in result section 4.1. "Virus induced over-expression of protein phosphatase 2A inhibits insulin signaling in chronic hepatitis C" are described there. Additional material and methods are described below.

Reagents, Antibodies and Cells

LXRα antibody was from R&D Systems Europe (Oxon, United Kingdom), LXRβ antibody from Abcam (Cambridge, United Kingdom).
Moloney murine leukemia virus reverse transcriptase, random hexamers and deoxynucleoside triphosphate were from Promega Biosciences Inc. (Wallisellen, Switzerland). qRT-PCR primers were synthesized by Microsynth, SYBR-Green PCR Master Mix was obtained from Applied Biosystems (Foster City, CA).
HA-PP2A cells are Huh7 derived cells stably expressing a HA-tagged PP2A catalytic subunit (PP2Ac). They are grown in DMEM, 10% fetal bovine serum, supplemented with penicillin/ streptomycin and 800 µg/ml of G418. Glucose free DMEM was obtained from Invitrogen (Lubioscience, Lucerne, Switzerland).

Quantitative RT-PCR

Total RNA extraction from human liver biopsy samples was done using NucleoSpin RNA II Kit (Macherey-Nagel, Oensingen, Switzerland) for mouse liver and cultured cells or RNAeasy mini Kit (Quiagen, Basel, Switzerland) for human liver biopsies according to manufacturer's instructions. RNA was reverse transcribed by reverse transcriptase in the presence of random hexamers and deoxynucleoside triphosphate. The reaction mixture was incubated for 5 minutes at 70°C and then for 1 hour at 37°C and was stopped by heating at 95°C for 5 minutes.
The cDNA was used to perform SYBR-PCR based on SYBR-Green-Fluorescence. To prevent influence from genomic DNA amplification, the primers were designed across exon-intron junctions. All reactions were run in duplicate using the ABI 7000 Sequence Detection System (Applied Biosystems). Relative mRNA expression levels were calculated from Δct values. The Δct value was derived by subtracting the threshold cycle (CT) value for GAPDH, which served as an internal control, from the CT values for the different genes.

Human primers:

GAPDH_forward	5'-GCTCCTCCTGTTCGACAGTCA-3'
GAPDH_reverse	5'-ACCTTCCCCATGGTGTCTGA-3'
LXRα_forward	5'- CCCTTCAGAACCCACAGAGATC -3'
LXRα_reverse	5'- CCCACACACGCTGCATAGC -3'
LXRβ_forward	5'- AAGAGGAGGACGAAGAAAAGCA -3'
LXRβ_reverse	5'- CCTCTCGCGGAGTGAACTACTC -3'
CAR_forward	5'- CACATGGGCACCATGTTTGA -3'
CAR_reverse	5'- AAGGGCTGGTGATGGATGAA -3'
PGC1α_forward	5'- CAAATGCACCTCCAAAAAGAAGTC -3'
PGC1α_reverse	5'- TTGTTGGTTTGGCTTGTAAGTGTT -3'
PEPCK_forward	5'- CGCCATGCGCTCTGAGT -3'

PEPCK_reverse	5'- GGGTCGTGCATGATGATCTTC -3'
G6PAse_forward	5'- GCTGCTCATTTTCCTCATCAAGT -3'
G6Pase_reverse	5'- AAAGTTTCTGCAACAGCAATGC -3'
GLUT2_forward	5'- TATCAGGACTATATTGTGGGCTAATTTC -3'
GLUT2_reverse	5'- AGAGCGGTTGGAGCAATTTC -3'

Mouse primers:

GAPDH_forward	5'- AAAGTGGACATTGTTGCCATCAAC -3'
GAPDH_reverse	5'- GTTCACACCCATCACAAACATGGG -3'
LXRα_forward	5'- GAGTGTCGACTTCGCAAATGC -3'
LXRα_reverse	5'- TCAAGCGGATCTGTTCTTCTGA -3'
LXRβ_forward	5'- CCCCACAAGTTCTCTGGACACT -3'
LXRβ_reverse	5'- TGACGTGGCGGAGGTACTG -3'
CAR_forward	5'- CATTGCGGCGAGCCA -3'
CAR_reverse	5'- GCTGATTCAGTTGCAAAGATGC -3'
PGC1α_forward	5'- AGCCGTGACCACTGACAACGAG -3'
PGC1α_reverse	5'- GCTGCATGGTTCTGAGTGCTAAG -3'
PEPCK_forward	5'- CATAACGGTCTGGACTTCTCTGC -3'
PEPCK_reverse	5'- GAATGGGATGACATACATGGTGCG -3'
G6PAse_forward	5'- ATGAACATTCTCCATGACTTTGGG -3'
G6Pase_reverse	5'- GACAGGGAACTGCTTTATTATAGG -3'

Fasting glucose, insulin and HOMA in mice

Mice were fasted o/n. Glucose was measured in mice fasted o/n from tail vein blood by Ascensia Contour blood glucose monitoring system (Bayer AG Health Care, Zurich, Switzerland). Insulin was measured from 5µl serum using Mercodia Ultrasensitive Mouse Insulin ELISA (Mercodia, Uppsala, Sweden). HOMA-IR was calculated by the formula: fasting glucose (mmol/l) x fasting insulin (mIU/l) /22.5 (13).

Glucose and insulin tolerance tests in mice

For the glucose tolerance test, mice were fasted o/n and glucose solution in saline was injected i.p. at 2g/kg body weight. Glucose was measured from tail vein blood before and 15, 30, 45, 60, 90 and 180 minutes after injection using Ascensia Contour blood glucose monitoring system.

For insulin tolerance test, mice were fasted for 4 hours and insulin solution was injected at 0.8 IU/kg body weight. Glucose was measured from tail vein blood before and 15, 30, 45, 60 and 90 minutes after injection.

The Basel NAFLD cohort study

The NAFLD cohort study is a prospective, long-term single center cohort study and has been established at the Department for Gastroenterology and Hepatology of the University Hospital in Basel in March 2003. Since December 2007 a second center was built up at the University Hospital in Zurich. Only patients with the histological diagnosis of NAFLD are recruited.

The aim of the study is to do a long-term observation of patients with NAFLD with or without NASH with the documentation of relevant anamnestic, physiologic, biochemical and histological parameters. This set of data might help to find out the number of patients that

progress from NAFLD to NASH or from NASH to NASH cirrhosis, respectively. Further to detect patients at risk for a progressive form of the disease. Finally to build up a serum-, plasma- and biopsy bank which enables us to investigate the use of emerging new tools.
Patients are seen once per year. The visits include personal and family anamnesis for diseases of the metabolic syndrome, dietary habits, sports, drugs and alcohol intake. Patients are examined focusing on blood pressure, heart rate, weight, BMI, waist circumference. Further a fasting blood sample is taken for analysis of glucose, insulin, c-peptide, triglycerides, cholesterol, AST, ALT, GGT, AP, bilirubin, albumin, INR, hematology. An oral glucose tolerance test is performed in patients without diagnosis of diabetes mellitus. α-fetoprotein is measured in patients with cirrhosis. By abdominal sonography, liver, pancreas and spleen are examined with a focus on steatosis of the liver, pancreatic lipomatosis and visceral fat accumulation.
Cytokeratin-18 fragments were measured using the M30-Aptosense ELISA Kit, Alexis Biochemicals (Grünwald, Germany).

Statistical analysis

Box plot diagrams, Mann Whitney tests, t-tests and Spearman correlations were performed using GraphPad Prism version 4.00 for Macintosh, GraphPad Software, San Diego California USA. Multivariate regression analysis was done using STATA/SE software, StataCorpLP, Texas, USA.

4. Results

1. Virus induced over-expression of protein phosphatase 2A inhibits insulin signaling in chronic hepatitis C

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Virus-induced over-expression of protein phosphatase 2A inhibits insulin signalling in chronic hepatitis C☆

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***Background/Aims*: Hepatitis C virus (HCV) infection disturbs glucose and lipid metabolism contributing to the development of liver steatosis, insulin resistance and type 2 diabetes mellitus. On the other hand, insulin resistance and steatosis have been found to be associated with increased rates of fibrosis progression and lower rates of response to interferon therapy in chronic hepatitis C (CHC). The molecular mechanisms contributing to insulin resistance in CHC are not well understood. We have shown previously that protein phosphatase 2A (PP2A) is over-expressed in biopsies from patients with CHC. In this study, we tested if PP2A over-expression leads to insulin resistance.**

***Methods*: We studied insulin signalling in cell lines that allow the regulated over-expression of HCV proteins and of the PP2A catalytic subunit (PP2Ac). Insulin signalling and PP2Ac expression were also studied in HCV transgenic mice and in liver biopsies from patients with CHC.**

***Results*: Over-expression of PP2Ac in cells inhibited insulin signalling by dephosphorylation of PKB/Akt. PP2Ac over-expression and impaired insulin signalling were found in the liver of HCV transgenic mice and in liver biopsies of patients with CHC.**

***Conclusions*: HCV-induced over-expression of PP2A in the liver contributes to the pathogenesis of insulin resistance in patients with CHC.**

***Keywords*: Viral hepatitis; Insulin resistance; Liver steatosis; Interferon**

1. Introduction

Chronic hepatitis C virus (HCV) infection is associated with liver steatosis, insulin resistance and type 2 diabetes mellitus. There is growing evidence that the virus directly causes insulin resistance through interference with the insulin signalling pathway and that HCV-induced dysregulation of important regulators of lipid metabolism such as sterol regulatory element binding protein (SREBP-1c) contributes to liver steatosis [1–10]. On the other hand, insulin resistance and steatosis have been found to be associated with increased rates of fibrosis progression and lower rates of response to interferon therapy in chronic hepatitis C (CHC) [5,11–16].

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Abbreviations: AMPK, adenosine monophosphate-activated protein kinase; CHC, chronic hepatitis C; GSK-3β, glycogen synthase kinase-3 beta; IRS-1, insulin-receptor substrate 1; mTOR, mammalian target of rapamycin; PDK1, phosphoinositide-dependent kinase 1; PP2A, protein phosphatase 2A; S6K1, S6 kinase 1.

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Insulin exerts its effects through binding to the insulin receptor, a receptor tyrosine kinase. The activated receptor then phosphorylates tyrosine residues on at least nine intracellular substrates including insulin-receptor substrate 1 (IRS-1), IRS-2 and Cbl [17,18]. The phosphorylated tyrosines in these substrates provide "docking sites" for proteins that contain src-homology-2 (SH2) domains, such as Grb2, SHP2, Fyn and the p85 regulatory subunit of phosphatidylinositol-3-OH kinase (PI(3)K). PI(3)K has a central role in the metabolic actions of insulin [19]. PI(3)K catalyses the generation of phosphatidylinositol-(3,4,5)-trisphosphate (PIP3), which activates phosphoinositide-dependent kinase 1 (PDK1). PDK1 together with mTORC2 complex activates the important serine/threonine kinase Akt/PKB [20,21]. Akt/PKB is activated by phosphorylation on threonine 308 and serine 473 [22]. Akt/PKB is a central regulator of the function of many cellular proteins that are involved in metabolism, survival, apoptosis, differentiation and proliferation [23]. Amongst these proteins are mTOR (mammalian target of rapamycin), S6K1 (S6 kinase 1) and GSK-3β (glycogen synthase kinase-3) [24], all of which are important for the control of glucose metabolism. In general, the activation of these proteins leads to enhanced glucose transport and the synthesis of glycogen and proteins.

Contrary to the activating effect of tyrosine phosphorylation, serine phosphorylation of IRS proteins inhibits insulin signalling. This inhibitory phosphorylation is part of a negative feedback to insulin signalling, because insulin activates a number of serine/threonine kinases including mTOR, S6K1 and atypical isoforms of protein kinase C (PKCζ and PKCλ) that mediate serine phosphorylation of IRS proteins [18,25]. Moreover, serine phosphorylation of IRS serves as a mechanism for cross-talk from other pathways that induce insulin resistance. Excess nutrients, fatty acids, pro-inflammatory cytokines (for example tumor necrosis factor α, TNFα, and interleukin-6, IL-6), the adipocyte secreted hormone resistin, and endosplasmic reticulum stress could at least in part exert their negative effect on insulin signalling through the activation of serine/threonine kinases such as c-Jun NH_2-terminal kinase (JNK), stress-activated protein kinases, and PKCθ [25–30].

Impaired IRS-1 and PI(3)K signalling has also been implicated in the pathogenesis of HCV associated insulin resistance and type 2 diabetes [31]. Insulin-induced IRS-1 tyrosine phosphorylation (but not IRS-1 content) was found to be decreased by 2-fold in liver biopsies from patients with CHC compared to controls [31]. Another group reported a decrease in IRS-1 and IRS-2 expression in liver biopsies from HCV patients and in HCV core transgenic mice [32], and more recently, that successful treatment of patients with CHC improves insulin sensitivity and increases IRS-1 and IRS-2 expression in the liver [33].

We had reported previously that HCV induces the over-expression of the serine/threonine phosphatase PP2A (protein phosphatase 2A) through an endoplasmatic reticulum (ER) stress response pathway [34], and that PP2A over-expression inhibits interferon α signalling in liver cells [35,36]. Interestingly, PP2A dephosphorylates and inactivates Akt/PKB in mouse fibroblasts [37]. Furthermore, PP2A was shown to negatively regulate insulin signalling in 3T3-L1 adipocytes [38]. We therefore investigated if HCV-induced over-expression of PP2A could be a molecular pathway linking HCV infection to insulin resistance.

2. Materials and methods

2.1. Reagents and antibodies

Human insulin, okadaic acid and wortmannin were from Sigma (Fluka Chemie, GmbH, Buchs, Switzerland). Purified PP2Ac and PP2Ac-, p85PI3K- and IRS-1- antibodies were from Upstate (LucernaChem, Luzern, Switzerland). Antibodies against pS473Akt, Akt, pT308Akt, pS9GSK3β, pThr172AMPKα and AMPKα were from Cell Signalling (Bioconcept, Allschwil, Switzerland), β-Actin from Sigma and p-Y-99 from Santa Cruz (LabForce AG, Nunningen, Switzerland). Secondary antibodies IRDye 800CW and IRDye 680 were from LI-COR Bioscience (Bad Homburg, Germany). The HCV core antibody was a gift from Darius Moradpour.

2.2. Patients and biopsies

Study patients were recruited from September 2005 to July 2007. The study was approved by the local ethics committee, and written informed consent was obtained. After removal of a 20- to 25-mm long biopsy specimen for routine histopathological workup for grading and staging of their liver disease according to Metavir [39] and a semi-quantitative assessment of liver steatosis (percent hepatocytes with lipid accumulation), the remaining biopsy specimens were treated with insulin and used for whole cell extraction and Western blotting. Patient characteristics and laboratory values are shown in Table 1. All samples that were treated with insulin *ex vivo* have a number written in bold. The controls are marked by a C in the column "genotype". They were obtained from patients that underwent ultrasound-guided biopsies of focal lesions in otherwise healthy livers (as assessed by histopathology and serum liver values).

From November 2006 to July 2007, fasting glucose and insulin were measured on the days of the biopsy. Insulin resistance was assessed by the HOMA score (homeostasis model assessment, calculated as (fasting insulin × fasting glucose)/22.5). During this period, 38 non-diabetic patients with chronic hepatitis C could be included in Basel. To increase statistical power, 34 additional patients were recruited at the University Hospital of Geneva, Switzerland, between August 2002 and December 2006: these were part of a larger series included in the HCV-MAID database [14], from which they were selected based on the availability of a frozen liver sample, optimal quality of tissue RNA and absence of diabetes. In Table 1, samples with HOMA scores and PP2Ac mRNA expression values have italic numbers, and samples from Geneva have a G in front of the number.

2.3. Mice

B6HCV mice constitutively express HCV proteins in hepatocytes. They were a gift of Marco Tripodi and Nicola La Monica and were described previously [40,41]. Animal experiments were approved by the veterinary office of the Kanton Basel.

Table 1
Liver biopsy and patient data

Nr[a]	Age	Sex	Geno-type[b]	Viral load (log IU/ml)	Steatosis (%)	Grade Metavir	Stage Metavir	Glucose (mmol/l)	Insulin (mIU/l)	HOMA	PP2Ac (protein ng/μg)	PP2Ac (mRNA dct)
10	40	m	4	6.32	0	2	3			ND	9.19	
13	27	m	3	4.94	0	1	1			ND	7.66	
14	77	m	ND	ND	<5	2	4			ND	5.21	
16	52	m	1	6.75	40	1	1			ND	6.89	
18	68	f	C	C	3	–	–			ND	3.72	
20	45	m	1	6.41	30	1	1			ND	12.67	
24	58	f	1	5.19	40	1	1	4.7	7.60	1.59	10.55	
26	44	m	3	5.81	10	1	2			ND	11.85	
27	28	m	3	5.49	0	1	0			ND	7.21	
28	41	m	4	ND	0	1	4			ND	7.66	
32	46	m	1	5.84	70	1	4			ND	9.89	
34	78	m	ND	ND	0	2	4			ND	8.51	
35	27	m	3	7.39	10	1	0			ND	6.21	
38	21	m	1	5.66	0	1	1			ND	12.72	
39	46	f	4	6.24	0	4	3	4.7	12.20	2.55	7.58	4.73
40	46	m	1	6.75	5	2	4	4.5	15.30	3.06	6.46	5.96
41	44	m	3	6.47	0	1	3			ND	6.91	
44	43	m	4	5.28	90	1	2			ND	8.47	
45	61	f	C	C	0	–	–			ND	4.39	
47	37	m	3	6.17	10	1	1			ND	7.93	
48	68	m	1	6.33	0	1	2			ND	11.00	
49	38	m	3	5.39	0	2	3			ND	8.67	
50	54	f	C	C	0	–	–			ND	4.17	
51	47	f	C	C	0	–	–			ND	2.71	
54	48	m	3	5.53	0	2	4			ND	3.75	
55	63	m	1	5.07	30	1	1			ND	4.51	
57	77	m	C	C	0	–	–			ND	4.34	
58	40	m	1	6.16	0	1	1			ND	4.96	
59	55	m	1	5.90	0	1	1			ND	3.47	
60	35	m	3	6.06	50	2	2			ND	4.46	
64	32	f	3	5.98	0	1	1			ND	2.95	
65	28	f	3	5.63	10	2	1			ND	2.79	
66	34	m	1	6.09	0	1	1			ND	3.14	
67	61	m	3	5.98	20	3	4			ND	10.13	
69	69	m	1	6.26	10	2	2			ND	7.53	
70	47	m	1	7.84	0	1	2			ND	6.64	
71	61	m	1	6.88	10	2	3			ND	7.21	
73	46	f	3	6.81	0	1	2			ND	5.10	
74	47	f	1	6.16	40	2	2			ND	4.76	
75	39	f	1	9.13	0	1	1			ND	3.02	
76	48	m	1	5.85	15	3	4			ND	6.41	
78	42	m	1	6.55	0	1	1			ND	4.62	
79	30	m	1	5.58	10	2	2			ND	3.99	
81	54	f	3	6.82	70	1	2			ND	8.08	
83	43	m	1	5.90	30	1	1			ND	7.10	
84	40	f	1	5.48	0	1	1			ND	5.71	
85	43	f	1	6.11	0	1	2			ND	7.35	
87	35	m	1	4.25	20	1	2			ND	6.17	
88	37	m	3	4.90	0	1	2			ND	7.29	
89	46	m	1	5.88	0	2	2	3.9	2.00	0.35	10.39	3.48
90	48	f	1	5.98	30	3	4	5.1	–	ND	6.86	5.25
91	52	f	1	6.65	5	2	2	5.6	8.70	2.17	5.50	5.12
92	38	m	1	6.98	0	2	1	5.5	12.80	3.13	5.31	6.17
93	63	f	2	6.52	30	2	3	4.8	9.90	2.11	5.76	5.87
94	38	f	2	6.76	0	2	3	4.3	3.10	0.59	3.95	5.49
95	41	m	1	7.09	0	2	3	5.4	28.00	6.72	4.72	5.23
97	53	f	2	6.08	0	3	3	5.1	14.20	3.22	5.00	5.44
98	61	m	4	3.94	0	3	4	5.1	18.20	4.13	1.43	5.53
99	59	m	1	5.28	10	3	3	5.1	10.80	2.45	5.08	4.44
100	33	m	2	6.27	0	1	2	5.6	4.90	1.22	3.66	6.04
101	41	f	1a	6.66	0	2	1	4.7	7.80	1.63	3.86	5.36

(*continued on next page*)

Table 1 (*continued*)

Nr[a]	Age	Sex	Geno-type[b]	Viral load (log IU/ml)	Steatosis (%)	Grade Metavir	Stage Metavir	Glucose (mmol/l)	Insulin (mIU/l)	HOMA	PP2Ac (protein ng/μg)	PP2Ac (RNA dct)
102	45	m	3a	6.01	10	2	1	5.5	5.80	1.42	3.44	5.96
103	47	m	2b	6.67	<5	3	4	5.6	25.70	6.40	3.41	5.40
104	48	f	4	4.95	0	1	1	4.4	3.10	0.61	5.49	5.14
105	63	m	1b	6.24	30	3	4	5.8	15.10	3.89	4.18	5.36
107	49	m	3a	6.11	20	1	2	4.7	4.50	0.94	4.03	5.72
108	58	m	2b	6.69	30	2	3	4.9	12.40	2.70	5.57	5.68
109	33	m	3a	6.23	5	2	1	4.7	3.70	0.77	6.02	5.82
110	53	f	2a/2c	4.86	50	2	4	5	13.40	2.98	4.42	5.68
112	43	f	3a	5.50	10	2	2	4.4	4.10	0.80	5.01	5.07
113	50	m	4e	5.93	50	3	4	5.9	17.80	4.67	2.91	5.53
114	56	m	1a	7.01	0	1	1	5.1	11.10	2.52	3.56	5.44
116	56	m	1b	6.89	0	2	3	6	9.60	2.56	2.99	5.79
117	32	f	1a	5.41	0	1	1	4.6	18.20	3.72	1.18	5.31
118	38	f	1b	6.90	0	2	3	4.9	3.50	0.76	2.82	5.11
119	53	f	1	ND	30	3	4	5.1	17.90	4.06	6.37	5.58
123	48	f	3a	7.13	70	2	2	4.6	4.50	0.92	2.36	6.08
125	35	m	1a	6.53	0	1	1	5.6	10.40	2.59	2.35	5.76
126	26	m	1b	7.12	<5	2	2	4.9	8.80	1.92	4.55	5.68
127	44	m	1b	4.83	0	2	4	5.5	18.20	4.45	3.67	5.51
130	24	f	1b	2.64	0	2	2	4.4	6.80	1.33	3.76	5.51
135	48	m	1a	7.56	0	1	2	4.6	8.60	1.76	2.38	6.12
136	48	m	ND	ND	80	1	1	4.2	5.20	0.97	3.16	6.63
138	31	m	ND	6.06	10	2	2	4.5	9.50	1.90	3.27	4.73
139	34	m	4c/4d	5.72	20	2	2	5.4	10.80	2.59	2.32	5.02
140	35	m	1a	5.35	10	1	1	5.4	18.20	4.37	2.18	5.96
G1	36	m	2a/2c	6.58	0	1	1	4.4	13.63	2.67	–	5.50
G2	64	f	1b	5.98	30–60	2	4	5.1	17.74	4.02	–	4.80
G3	36	m	1b	5.49	0	1	1	5.1	14.01	3.18	–	6.11
G4	31	m	1b	6.47	5–30	0	0	4.7	21.97	4.59	–	5.86
G5	48	m	4	5.92	0	2	1	4.3	8.90	1.70	–	6.39
G6	39	m	4	5.17	5–30	2	4	6.3	32.96	9.23	–	5.74
G7	34	m	3a	6.61	30–60	2	1	5.1	15.72	3.56	–	6.08
G8	46	m	3a	5.51	5–30	1	1	4.6	13.77	2.82	–	5.73
G9	35	m	1a	6.46	0	1	3	4	20.14	3.58	–	4.23
G10	41	m	4e	5.26	0	0	0	4.9	16.16	3.52	–	6.05
G11	34	m	1a	6.60	5–30	2	1	4.2	22.44	4.19	–	5.64
G12	31	m	1b	6.46	–	–	–	5.3	14.63	3.45	–	5.95
G13	35	m	1b	4.16	–	–	–	4.3	8.97	1.71	–	5.36
G14	51	f	2c	6.74	–	–	–	4.9	11.22	2.44	–	5.30
G15	53	m	2c	6.11	–	–	–	5.3	18.22	4.29	–	5.30
G16	31	f	1a	6.27	0	1	1	4.9	14.46	3.15	–	4.41
G17	62	f	2c	6.57	5–30	0	1	4.3	16.09	3.07	–	5.62
G18	35	f	1b	5.46	30–60	0	0	5.7	35.62	9.02	–	4.98
G19	31	m	1b	5.91	0	1	1	5	10.98	2.44	–	5.20
G20	43	f	1b	5.00	–	–	–	4.7	19.61	4.10	–	4.84
G21	57	m	1	6.10	–	–	–	6	24.64	6.57	–	5.37
G22	33	m	4	6.43	5–30	0	1	4.7	8.37	1.75	–	5.81
G23	49	m	2	6.14	0	1	1	4.2	12.68	2.37	–	6.83
G24	69	m	1	6.30	0	2	2	6.7	16.7	4.97	–	6.61
G25	31	m	1	5.26	0	1	1	4.7	13.7	2.86	–	6.45
G26	46	m	1	6.15	5–30	1	3	6	17.4	4.64	–	6.68
G27	36	m	ND	ND	5–30	1	2	2.8	10.71	1.33	–	4.45
G28	40	m	ND	5.77	0	1	1	5.5	8.6	2.10	–	5.28
G29	29	f	4	6.08	0	0	0	5.3	5.9	1.39	–	5.17
G30	49	m	1	6.96	0	1	1	5.5	12.3	3.01	–	4.65
G31	27	m	2	6.67	0	1	1	3.4	10.54	1.59	–	4.91
G32	45	m	3	ND	0	0	0	2.7	4.09	0.49	–	4.88
G33	25	m	1	ND	0	0	0	4.2	12.6	2.35	–	5.32
G34	46	m	1	5.48	0	1	0	4.6	6.71	1.37	–	4.58

[a] All samples that were treated with insulin *ex vivo* have a number written in bold. Samples with HOMA scores and PP2Ac mRNA expression values have italic numbers, and samples from Geneva have a G in front of the number.

[b] The controls are marked by a C in the column "geno-type".

2.4. Cell culture and transfection

UHCV-57.3 cells were a gift from Darius Moradpour and had been described previously [42].

UPP2A-C8 cells allow the inducible 2–3 times over-expression of the Cα subunit of PP2A. They were derived by transfecting the U-2 OS derived parental cell line UTA-6 [42] with a plasmid encoding the entire PP2Acα protein under the control of a tTA-dependent promoter [43].

2.5. PP2A phosphatase activity assay

PP2A activity was assessed according to the manufacturer's instructions (Promega, Wallisellen, Switzerland).

2.6. Preparation of extracts

UPP2A-C8 and UHCV-57.3 cells were starved from FCS and grown in low-glucose (5 mM) MEM overnight. They were stimulated with 100 nM human insulin in 25 mM Hepes at 37 °C for 5 min. Whole cell extracts were prepared as previously described [35]. Phosphatase inhibitor cocktail 1 for serine/threonine phosphatases (from Sigma) was added 1:100 to the lysis buffer. Mouse liver specimens were homogenized in 500 μl of lysis buffer and centrifuged at 18,000 rpm for 30 min. Biopsy specimen was incubated at 37 °C for 5 min in 25 mM Hepes (controls) or in 25 mM Hepes with 100 nM insulin. Biopsies were then homogenized in 100 μl lysis buffer. Lysates were centrifuged at 14,000 rpm for 5 min. Protein concentrations were determined with Bradford Assay (Bio-Rad Laboratories AG, Reinach, Switzerland).

2.7. Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were performed as previously described [35]. For detection of pY-IRS 1, IRS-1 and p85PI3K a wet transfer method (400 mA for 4 h, 7.5% methanol) was used and the membranes were blocked for 1 min with PVA blocking solution (25 mM Tris, pH 7.5, 150 mM NaCl and 1 μg/ml polyvinyl alcohol).

For quantification of PP2Ac expression in human liver biopsies and UPP2A-C8 cells, a standard curve was generated by loading 10, 25, 50 and 100 ng of purified PP2A on each gel. The intensity of each PP2Ac band was measured using NIH imaging software for UPP2A-C8 cells or the Odyssey Infrared Imaging System [44] for human biopsies.

2.8. Real-time RT-PCR

Total RNA extraction from human liver biopsy samples was done using NucleoSpin RNA II Kit (Macherey-Nagel, Oensingen, Switzerland). Quantification was done with qRT-PCR (SYBR-Green PCR Master Mix, Applied Biosystems, Foster City, CA). The primers for GAPDH were 5′-GCTCCTCCTGTTCGACAGTCA-3′ and 5′-ACCTTCCCCATGGTGTCTGA-3′, the primers for PP2Ac were 5′-CCACAGCAAGTCACACATTGG-3′ and 5′-CAGAGCACTTGATCGCCTACAA-3′. All reactions were run in duplicate using the ABI 7000 Sequence Detection System (Applied Biosystems). Relative PP2Ac mRNA expression levels were expressed as PP2AcΔct. The Δct value was derived by subtracting the threshold cycle (CT) value for GAPDH, which served as an internal control, from the CT value for PP2Ac.

2.9. Short hairpin siRNA inhibition of PP2Ac expression

Hairpin oligonucleotides against human PP2A catalytic subunit α isoform were designed using siRNA Designer Tool (Promega). Briefly, two hairpin oligonucleotides (Forward strand: 5′-ACCGGGATACCGTTTAATTTAATTCAAGAGATTAAATTA AACGGTATCCCTTTTTC-3′ and Reverse strand: 5′-TGCAGAAAAAGGGATACCGTTTAATTTAATCTCTTGAATTAAATTAAACGGTATC C-3′) were annealed to form a double-stranded DNA fragment. This DNA fragment was ligated into the psiSTRIKE Neomycin Vector (Promega). For PP2Ac silencing, Huh7 cells were transfected with 2 μg of plasmid using FUGENE HD (Roche) at the ratio 3:2. Twenty-four hours after the transfection, cells were selected with a medium containing 400 μg/ml of G418. Expression level of PP2Ac was checked after 5 days post-transfection.

2.10. In vitro dephosphorylation assay

Huh7 cells were stimulated with insulin or ionomycin, respectively, and lysed as described (but without orthovanadate and phosphatase inhibitor cocktail 1 in the lysis buffer). Fifty micrograms of whole cell extracts were incubated with 0.1 U and 0.25 U of purified PP2A and 7 μg of Bcl-2 (upstate) or BSA (Sigma) for 10 min at RT. The reaction was stopped by boiling the samples for 5 min. Phosphorylation of Akt and AMPK was determined by Western blot.

2.11. Statistical analysis

Box plot diagrams, *t*-tests and Spearman correlations were performed using GraphPad Prism version 4.00 for Macintosh, GraphPad Software, San Diego, CA, USA, www.graphpad.com.

3. Results

3.1. Expression of HCV proteins or PP2Ac in cells inhibits insulin signalling

To test if HCV protein expression inhibits insulin signalling, we made use of UHCV-57.3 cells that allow the regulated expression of HCV proteins [42]. Insulin-induced tyrosine phosphorylation of IRS-1 and IRS-1 association with PI3K were normal in cells expressing HCV proteins, but serine phosphorylation of Akt/PKB and GSK3β were impaired (Fig. 1A).

We had previously shown that the inhibitory effects of HCV protein expression on interferon α signalling were caused by HCV-induced PP2A over-expression [35]. We therefore used UPP2A-C8 cells to test if PP2Ac over-expression in the absence of HCV protein expression could also inhibit insulin signalling, and found the same effects on insulin signalling as in UHCV-57.3 cells (Fig. 1B and C).

3.2. Insulin signalling is impaired in HCV transgenic mice

B6HCV mice constitutively express HCV proteins in hepatocytes [41]. We had previously found that PP2Ac is over-expressed in the liver of B6HCV mice compared to C57BL/6 controls [35]. We therefore tested if insulin signalling is impaired in the liver of B6HCV mice. Mice were injected intraperitoneally with 2 IU insulin per kilogram body weight, and sacrificed 40 min later. As shown in Fig. 2, insulin-induced serine 473 phosphorylation of Akt/PKB was significantly inhibited in B6HCV mice.

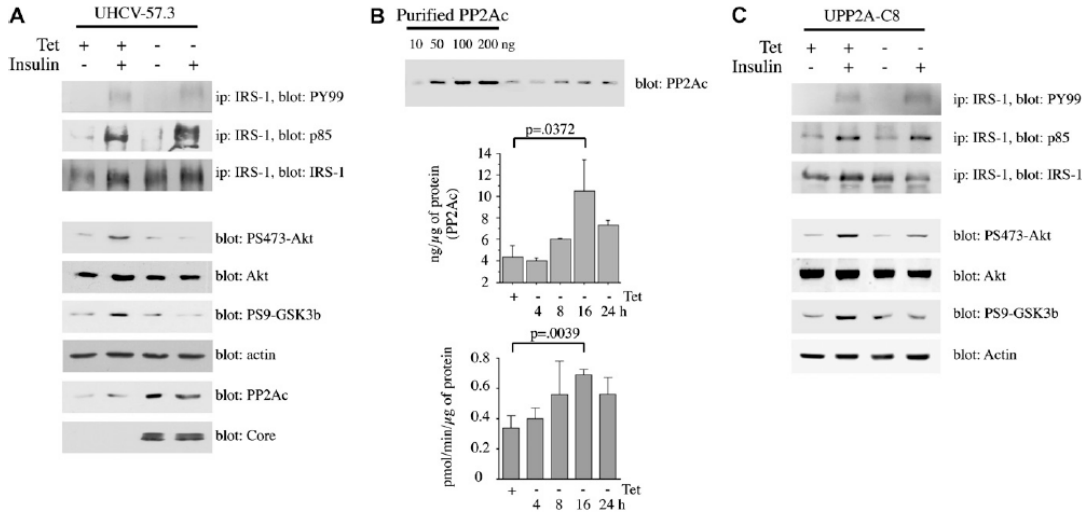

Fig. 1. Insulin signalling is inhibited in cells expressing HCV proteins (UHCV-57.3) or over-expressing PP2Ac (UPP2A-C8). (A) UHCV-57.3 cells were cultured in the presence (left two lanes) or in the absence (right two lanes) of tetracycline for 24 h. When grown in the absence of tetracycline, UHCV-57.3 express all viral proteins. As an example HCV core protein expression is shown in the lowest panel. As expected, viral protein expression induces the over-expression of PP2Ac (second last panel). Cells were then left untreated (lanes 1 and 3) or stimulated for 5 min with 100 nM insulin (lanes 2 and 4). After immunoprecipitation with anti-IRS-1, tyrosine phosphorylation of IRS-1 was tested with an antibody against phosphotyrosine (PY99, upper panel) and co-immunoprecipitation of the p85 subunit of PI(3)K with an p85 specific antibody (second panel). Both IRS-1 tyrosine phosphorylation and IRS-1-p85 association were unchanged by viral protein expression. The third panel is a loading control for IRS-1 protein. Phosphorylation of Akt/PKB on serine 473 (fourth panel) and of GSK3β on serine 9 (sixth panel) were tested with anti-phospho-serine473Akt and anti-phospho-serine9GSK3β, respectively. Viral protein expression strongly inhibited both Akt/PKB and GSK3β phosphorylation (lane 4 versus lane 2). (B) UPP2A-C8 cells allow the over-expression of the catalytic α subunit of PP2A (PP2Acα) when grown in the absence of tetracycline. To measure PP2Ac expression, whole cell extracts of UPP2A-C8 cells obtained after 4, 8, 16 and 24 h of culture in the absence of tetracycline were analysed by Western blot together with 10, 50, 100 and 200 ng purified PP2Ac (a representative example is shown in the upper panel). The purified PP2Ac samples were used to calculate a calibration curve. The middle panel shows the mean (±SEM) of three experiments. The maximum of PP2Ac over-expression (2.5-fold) was observed after 16 h. The phosphatase activity of PP2A was measured at the same time points with a commercially available phosphatase assay. The lower panel shows the mean (±SEM) of three experiments. The enzymatic activity increased up to 2-fold. (C) Insulin stimulated IRS-1 tyrosine phosphorylation, p85-IRS-1 association, Akt/PKB serine 473 phosphorylation and GSK3β serine 9 phosphorylation were analysed in UPP2A-C8 cells grown in the presence (lanes 1 and 2) and in the absence (lanes 3 and 4) of tetracycline (16 h). Stimulation of IRS-1 and association of IRS-1 with the p85 subunit of PI(3)K was not changed by PP2Ac over-expression (lanes 2 versus 4), but phosphorylation of Akt/PKB and GSK3β were consistently impaired.

3.3. PP2A constitutively inhibits Akt/PKB by serine 473 dephosphorylation

To test if PP2A could dephosphorylate Akt/PKB *in vitro* we added increasing amounts of purified PP2A to cell extracts of insulin stimulated Huh7 cells, and observed a dephosphorylation of Akt/PKB (Fig. 3A). This dephosphorylation could be inhibited by adding a serine/threonine phosphatase inhibitor mix to the cell extracts (Fig. 3B). Since over-expression of PP2A in UHCV-57.3 and UPP2A-C8 cells inhibited insulin-induced serine 473 phosphorylation (Fig. 1), we wondered if inhibition of PP2A in cells would result in Akt/PKB phosphorylation even in the absence of insulin stimulation. As shown in Fig. 3C, a pretreatment of the cells with 250 nM okadaic acid, an inhibitor of PP2A, resulted in a phosphorylation of Akt/PKB on serine 473 and threonine 308. When cells were pre-incubated with the PI3K inhibitor wortmannin, neither threonine 308 nor serine 473 phosphorylation of Akt/PKB was induced by okadaic acid treatment. An increase of serine 473 phosphorylation of Akt/PKB was also evident in cells where PP2Ac expression was reduced using a shRNA silencing vector (Fig. 3D). These data support a model where PP2A is necessary for the constant dephosphorylation and inactivation of Akt/PKB, thereby counteracting a constitutive phosphorylation through background activity levels of PI3K that are present even in the absence of exogenous stimuli such as insulin. We conclude that PP2A is an important negative regulator of insulin signalling.

3.4. Insulin signalling is impaired in liver biopsies from patients with chronic hepatitis C

Next, we analysed PP2A expression and insulin signalling in liver biopsies from patients with CHC. Parts of the biopsy material that were not used for routine his-

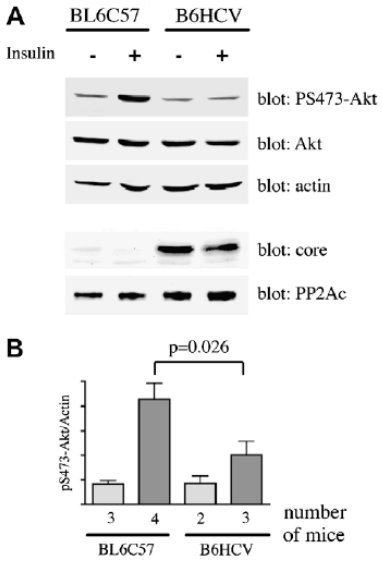

Fig. 2. Insulin signalling is inhibited in the liver of HCV transgenic (B6HCV) mice. (A) Representative panel of Western blots showing normal expression of Akt/PKB but strongly inhibited insulin stimulated phosphorylation of serine 473 of Akt/PKB. Animals were injected intraperitoneally with 2 IU/kg body weight insulin and sacrificed 40 min later. Western blots were performed with whole cell extracts from the liver. (B) Liver extracts from the indicated number of mice were used for statistical analysis. Quantification of pS473-Akt/PKB and actin signals was done with Odyssey Imaging Software. Shown are the means (±SEM). The difference between insulin treated control mice and HCV transgenic mice was statistically significant ($p = 0.0261$, t-test).

topathological evaluation were stimulated immediately *ex vivo* with 100 nM insulin or incubated in buffer for 5 min. Whole cell extracts were then tested for PP2Ac expression and activation of insulin signalling pathway components. Fifty-five biopsies from patients with CHC and five biopsies from controls were analysed. Table 1 shows their demographic data, laboratory values and the histopathological evaluation of the liver biopsies.

The controls were obtained from patients that underwent ultrasound-guided biopsies of focal lesions in otherwise healthy livers. There was a statistically significant over-expression of PP2Ac in liver biopsies from CHC patients (Fig. 4A and B). The median PP2Ac expression increased from 4.2 ng/μg total protein in controls to 6.5 ng/μg total protein in HCV patients. Since PP2Ac is a very abundant protein with a tight regulation of its expression, such an increase by more than 50% has biological relevance.

There was also a significant inhibition of insulin signalling in CHC biopsies. Insulin signalling was tested using a semi-quantitative Western blot analysis of serine 473 phosphorylation on Akt/PKB (Fig. 4C and D). It is

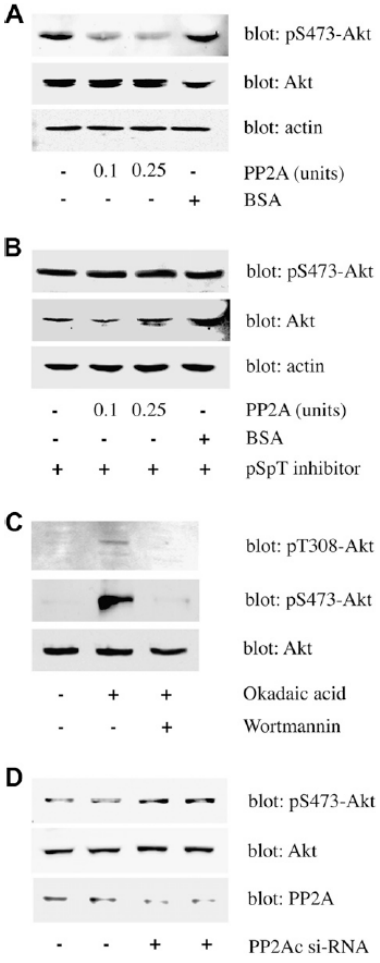

Fig. 3. PP2A regulates serine 473 phosphorylation of Akt/PKB. (A) Huh7 cells were stimulated with 100 nM insulin for 5 min. Whole cell extracts were incubated with purified PP2A (0.1 and 0.25 U) or BSA (7 μg) for 10 min at RT. Samples were immunoblotted for pS473Akt, Akt and actin. (B) Dephosphorylation of Akt/PKB is inhibited by serine/threonine phosphatase inhibitor cocktail 1. The experiment shown in (A) was repeated but a serine/threonine phosphatase inhibitor mix was added to all samples. (C) Inhibition of PP2Ac by okadaic acid induces phosphorylation of Akt/PKB. Huh7 cells were treated with 250 nM okadaic acid for 2 h (without insulin stimulation), and phosphorylation of Akt/PKB on threonine 308 (upper panel) and serine 473 (middle panel) was analysed by Western blot. A pre-incubation of Huh7 cells with 100 nM wortmannin (an inhibitor of PI3K) for 2 h together with 250 nM okadaic acid prevented the okadaic acid-induced Akt/PKB threonine 308 and serine 473 phosphorylation. (D) siRNA knockdown of PP2Ac leads to an increase in serine 473 phosphorylation of Akt/PKB. Huh7 cells were transfected with a shRNA vector targeting the expression of PP2Ac. Five days later, cell extracts were analysed by Western blots. Duplicate samples are shown for silenced cells and controls.

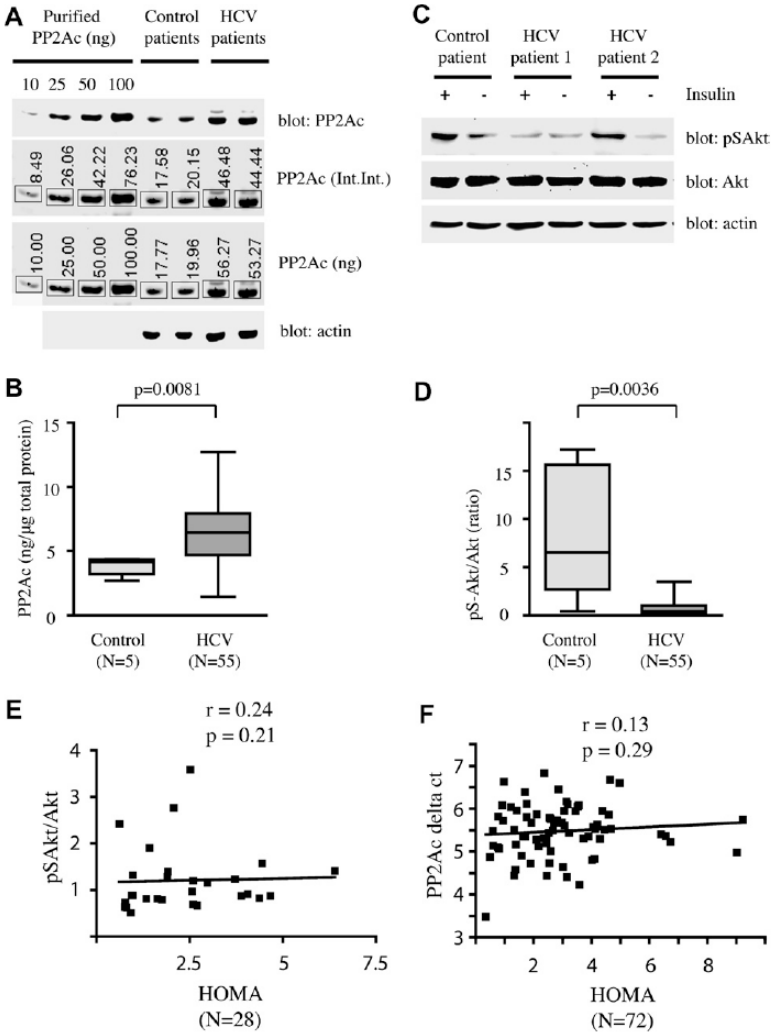

Fig. 4. PP2A is over-expressed and insulin stimulated serine 473 phosphorylation of Akt/PKB is reduced in liver biopsies of CHC patients. (A) Representative example of a Western blot showing samples with 10, 25, 50 and 100 ng of purified PP2A used to generate a calibration curve and samples of liver biopsy homogenate from control and HCV patients (same samples in all panels). The upper panel shows the Western blot, the second panel the integrated intensity (Int.Int.) of the PP2Ac Western blot bands measured by Odyssey Infrared Imaging System, and the third panel the concentration of PP2Ac in the liver biopsies as calculated using the calibration curve obtained with the purified PP2A samples and the integrated intensity values obtained from the Imaging System. The lower panel shows a Western blot of actin as a loading control. (B) Box plot diagram showing the PP2Ac expression levels in the liver biopsies of 5 control patients and 55 patients with chronic hepatitis C. PP2Ac expression is significantly higher in CHC than control patient's biopsies ($p = 0.0081$, Mann–Whitney *U* test). (C) Insulin-induced serine 473 phosphorylation of Akt/PKB is impaired in liver biopsies of CHC patients. Liver biopsies were stimulated *ex vivo* with 100 nM insulin for 5 min. Shown are three examples, a control patient with normal Akt/PKB phosphorylation (left), a HCV patient with defective signalling (patient 1) and a HCV patient with intact signalling (patient 2). (D) Integrated intensity values of pSAkt/PKB normalized to Akt/PKB in insulin treated liver biopsy specimens were calculated. The results are shown in a box plot diagram. The difference between controls and HCV patients is statistically significant ($p = 0.0036$, Mann–Whitney *U* test). (E) The ratio of phosphorylated Akt/PKB to total Akt/PKB was assessed by Western blots in 28 liver biopsies that had been treated for 5 min with 100 nM insulin *ex vivo*. There is no significant correlation between HOMA scores and Akt/PKB phosphorylation (Spearman $r = 0.244$, $p = 0.21$). (F) The expression levels of PP2Ac mRNA were measured in 72 biopsies of non-diabetic patients by real-time RT-PCR. Shown are the Δct values compared to the housekeeping gene GAPDH. The correlation between PP2Ac expression and HOMA is not significant (Spearman $r = 0.125$, $p = 0.29$).

noteworthy that insulin signalling was not impaired in all CHC patients. Fig. 4C shows an example of a CHC patient with normal insulin-induced serine 473 phosphorylation (HCV patient 2) and one of the

patients with defective insulin signalling (HCV patient 1). However, as a group, CHC patients had significantly impaired insulin signalling (Fig. 4D).

Systemic insulin resistance involves not only glucose metabolism in the liver, but also adipose tissue and muscle. To test if the inhibition of hepatic insulin signalling observed in patients with CHC correlates with systemic insulin resistance, we measured fasting glucose and insulin on the day of the liver biopsy. In a group of 28 non-diabetic patients where we could analyse phosphorylation of Akt/PKB induced by *ex vivo* stimulation with insulin, we could not find a significant correlation to HOMA (Fig. 4E). In a larger group of 72 patients we compared the expression levels of PP2Ac mRNA with their HOMA, but again, no significant correlation was present (Fig. 4F).

We also analysed if there are statistically significant correlations between PP2Ac expression and insulin-induced Akt/PKB phosphorylation with parameters such as gender, virus load, genotype, fibrosis stage, prior therapy and response to therapy. However, no such correlation was found (data not shown).

3.5. Adenosine monophosphate-activated protein kinase (AMPK) is inhibited by PP2A

AMPK is activated in response to ATP depletion and is a central component of a protein kinase cascade that plays a key role in the cellular response to low-energy [45]. AMPK can also be activated by phosphorylation of its α subunit on threonine 172 by upstream kinases such as LKB1 [46]. When activated, AMPK regulates a number of key enzymes of the intermediary metabolism with the general effect of shutting down ATP-consuming metabolic pathways and activating ATP-generating pathways [47]. In recent years it has become evident that AMPK is not only important for cellular energy balance, but also for systemic glucose homeostasis at the whole body level. AMPK enhances insulin sensitivity and might be a potential target of anti-diabetic drug therapies. We therefore were interested if the over-expression of PP2A in the liver of patients with CHC would have an effect not only on insulin signalling but also on the activation status of AMPK. First, we tested if PP2A is involved in the regulation of AMPK in Huh7 cells. As shown in Fig. 5A, the α subunit of AMPK (AMPKα) was found to be constitutively phosphorylated on threonine 172 in Huh7 cells. This phosphorylation was enhanced when cells were treated for 2 h with 250 nM okadaic acid. Furthermore, siRNA silencing of PP2Ac increased the phosphorylation on threonine 172 (Fig. 5B). Similar to Akt/PKB, AMPK seems to be continuously phosphorylated by upstream kinases and then dephosphorylated by PP2A either directly or indirectly. A role of PP2A in AMPK dephosphorylation is supported by the finding that addition of purified PP2A to whole cell extracts of Huh7 cells leads to a dephosphorylation of pThr172AMPKα (Fig. 5C).

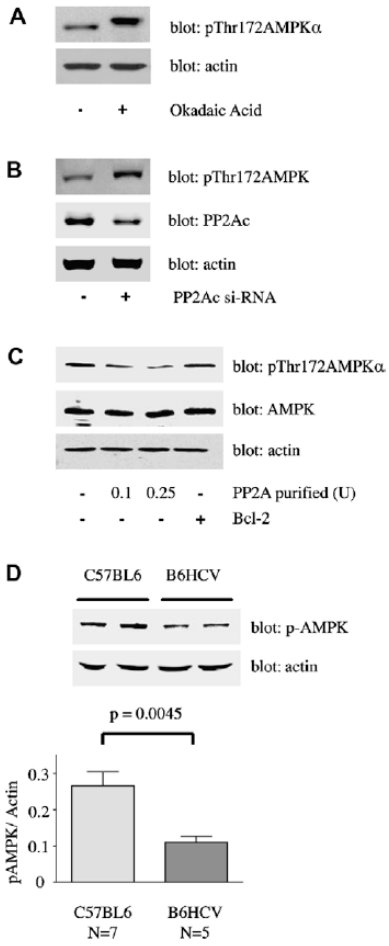

Fig. 5. AMPK is inhibited by PP2A. (A) Huh7 cell extracts were tested for phosphorylation of AMPKα on threonine 172 by Western blots. The constitutive phosphorylation of AMPK was strongly enhanced by blocking PP2A with 250 nM okadaic acid for 120 min. (B) siRNA knockdown of PP2Ac leads to an increase in threonine 172 phosphorylation of AMPK. Huh7 cells were transfected with a shRNA vector targeting the expression of PP2Ac. (C) Huh7 cells were stimulated for 5 min with 1 μM ionomycin to induce threonine 172 phosphorylation of AMPKα. The cell extracts were then incubated with 0.1 and 0.25 U of purified PP2A or with 7 μg of purified Bcl2 (as a control) for 10 min. The pThr172AMPKα signal was specifically reduced in the samples incubated with PP2A. (D) Constitutive threonine 172 phosphorylation of AMPK was tested in liver homogenates of HCV transgenic mice (B6HCV) and control mice (C57BL/6). The upper panel shows a representative Western blot with two mice from each group. Liver extracts from seven control mice and five B6HCV mice were analysed and used for statistics (lower panel). Quantification of pThr172AMPKα and actin signals was done with Odyssey Imaging Software. Shown are the means (±SEM). The difference between control mice and HCV transgenic mice was statistically significant ($p = 0.0045$, t-test).

Next, we tested AMPK phosphorylation in liver extracts of B6HCV mice. We have previously shown that B6HCV mice have a constitutive over-expression of PP2Ac in the liver [35]. Since PP2A phosphatase activity is also increased in B6HCV mice [35], we hypothesized that the phosphorylation of AMPKα would be decreased in these mice. Indeed, constitutive pThr172AMPKα signals were significantly weaker in liver homogenates of B6HCV mice (Fig. 5D), consistent with a negative regulatory role of PP2A on AMPK signalling.

4. Discussion

We had previously reported that HCV infection induces an over-expression of the important cellular phosphatase PP2A, and the PP2A over-expression *per se* inhibits IFNα signalling through the Jak-STAT pathway [34–36,40,48]. Here we show that PP2A over-expression also inhibits insulin signalling through the Akt/PKB signalling pathway. PP2A directly or indirectly dephosphorylates Akt/PKB on serine 473, and thereby lowers the kinase activity of Akt/PKB, with consequences on both gluconeogenesis and glycogen synthesis. One of the substrates of Akt/PKB is GSK3 (glycogen synthase kinase), a key enzyme of glycogen metabolism. Akt/PKB inactivates GSK3 through serine phosphorylation. This inactivation of GSK3 in turn results in an activation of glycogen synthase, and finally enhanced intracellular storage of glucose in glycogen. We report here an inhibition of insulin-induced GSK3β phosphorylation in cells expressing HCV proteins or over-expressing PP2A (Fig. 1). Thus PP2A over-expression in chronic hepatitis C could inhibit glycogen synthase activity, favouring release of glucose from cells into the blood. Another substrate of Akt/PKB is phosphorylase-a, an enzyme that is instrumental for glycogen synthesis in hepatocytes [49]. An inhibition of Akt/PKB by PP2Ac over-expression could result in an activation of phosphorylase-a and thereby a decreased glycogen synthesis in response to insulin.

PP2A not only affected insulin signalling, but also a second key player in glucose metabolism, adenosine monophosphate-activated protein kinase. AMPK has received much attention lately because it was found that the widely used anti-diabetic drug metformin exerts its effects through the activation of AMPK. AMPK is activated by phosphorylation and inhibits gluconeogenesis through the regulation of a number of intermediary signalling pathways. In the present paper we report that the constitutive level of AMPK phosphorylation is reduced in the liver of HCV transgenic mice that over-express PP2A in hepatocytes. Furthermore, addition of purified PP2A to cell extracts resulted in a dephosphorylation of AMPK, whereas pharmacological inhibition of PP2A with okadaic acid or PP2Ac silencing strongly increased the AMPK phosphorylation. We conclude that HCV-induced PP2A over-expression contributes to insulin resistance by inhibiting not only insulin signalling at the level of Akt/PKB but also AMPK.

When we correlated the liver biopsy data with the HOMA index, we did not find a statistically significant correlation between insulin signalling in liver biopsies and systemic insulin resistance. However, besides the liver, also the adipose and muscle tissue play an important role in the pathogenesis of systemic insulin resistance. Both of these tissues are not infected with HCV, but insulin sensitivity in them will show a considerable inter-individual variation. Therefore it is likely that very large numbers of patients would have to be investigated to detect a statistically significant correlation between hepatic PP2Ac over-expression and systemic insulin resistance.

It has been reported that the prevalence of liver steatosis is increased in patients with CHC [5,50,51]. In genotype 3 infections, a direct cytopathic effect of the virus seems to be responsible for fat accumulation in hepatocytes [5,52], whereas in patients infected with non-genotype 3, insulin resistance seems to be central for the pathogenesis of liver steatosis [2]. In accordance with these data, we found a weak but statistically significant negative correlation between insulin-induced Akt/PKB phosphorylation and the degree of steatosis (percentage of hepatocytes with lipid accumulation) in liver biopsies of patients with non-genotype 3 infections (Spearman correlation, $N = 40$, $r = -0.4202$, $p = 0.0035$). There was also a weak correlation between PP2A protein expression levels and the degree of steatosis in biopsies from non-genotype 3 infected patients (Spearman correlation, $N = 40$, $r = 0.2373$, $p = 0.0702$).

Endoplasmic reticulum (ER) stress has been linked to impaired insulin signalling and insulin resistance [28], providing a rationale for targeting the ER stress response pathways for the treatment of type 2 diabetes [53]. Interestingly, we have recently reported that HCV infection induces ER stress and that ER stress *per se* results in an up-regulation of PP2A [34]. Moreover, PP2A enhances the helicase activity of HCV NS3, thereby promoting viral replication [48]. Thus, PP2A up-regulation during HCV infection might be a central component of the virus–host interaction that contributes not only to viral persistence and to treatment failures of peglFNα therapies, but also to the pathogenesis of insulin resistance and liver steatosis in CHC.

Acknowledgements

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2. *Overexpression of PP2A dysregulates glucose induced gene regulation – a role in Liver X receptor (LXR) regulation?*

LXR in PP2Ac expressing cells (Huh7 vs. HA-PP2A cells)

Glucose metabolism in the liver is regulated not only by insulin but also by glucose itself.
It has been shown recently, that glucose is a ligand at the LXRα and –β receptors and inhibits expression of gluconeogenic genes (110).
First we analysed mRNA and protein expression of LXRα and LXRβ in Huh7 and in Huh7 cells stably transfected with a constitutive active PP2Ac (HA-PP2A cells) by quantitative RT-PCR and Western Blot. There was no significant difference in mRNA or protein expression of both nuclear receptors (Figure 3A).
To test whether glucose induced inhibition of gluconeogenic genes is affected by PP2A, Huh7 and HA-PP2A cells were grown in glucose free medium and treated with glucose 20mM overnight. Gene expression of PGC1α, PEPCK and G6Pase was analysed by quantitative RT-PCR. We observed that inhibition of PGC1α and PEPCK was attenuated in HA-PP2A cells. Unexpectedly G6Pase was not inhibited but upregulated by glucose treatment in both cell lines. This upregulation was again attenuated in HA-PP2A cells (Figure 3B). These data suggest that PP2A negatively regulates glucose induced and presumably LXR dependent regulation of gluconeogenesis.

Figure 3

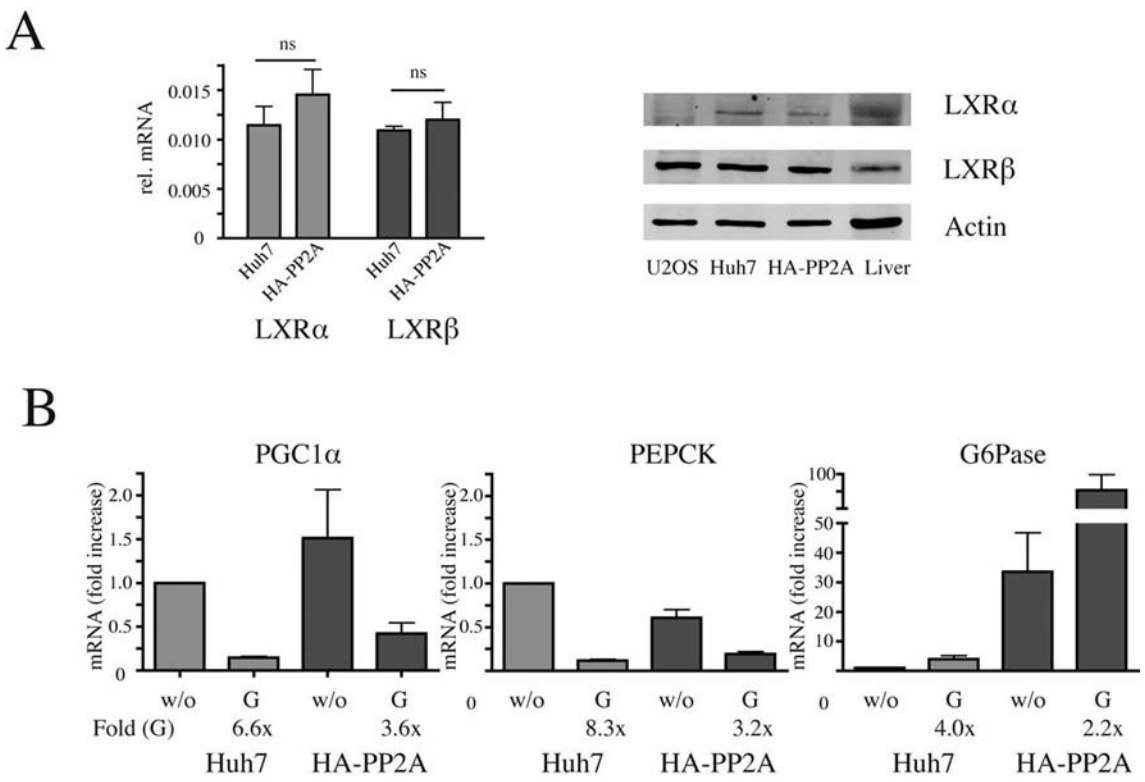

Figure 3: PP2A does not influence LXR expression but glucose induced gene regulation by LXR.

A) *PP2A does not influence mRNA- and Protein expression of LXRα and LXRβ in Huh7 and HA-PP2A cells.* LXRα and LXRβ mRNA expression was assessed by quantitative RT-PCR from 3 independent experiments from Huh7 and HA-PP2A RNA-extracts. Relative mRNA is shown as the number of copies relative to GAPDH (left), statistical analysis was done using a two-tailed unpaired t-test. Protein levels were analysed by Western Blot from whole cell extracts from Huh7 and HA-PP2A cells, from U2OS cells as a negative control and from a homogenized sample of human liver biopsy as a positive control. Shown is a representative Western Blot for 2 independent experiments (right).

B) *Glucose induced regulation of gluconeogenic key enzymes is impaired in HA-PP2A cells.* Huh7 and HA-PP2A cells were grown in glucose free medium and stimulated with glucose 20mM overnight (o/n). Quantitative RT-PCR for indicated genes was done in duplicate in 3 independent experiments. Relative mRNA expression is shown as a fold increase relative to the Huh7 untreated sample. Fold inhibition (for PGC1α and PEPCK) or fold increase (for G6Pase) of gene expression by glucose in Huh7 and HA-P2A cells is indicated as Fold (G).

LXR in vivo

To investigate nuclear receptor expression in vivo, we further analysed mRNA expression of LXRα, LXRβ and CAR in the liver of C57BL6 and B6HCV mice. Mice were starved for 8 hours. We found LXRα and CAR significantly lower expressed in B6HCV mice whereas LXRβ mRNA expression did not differ (Figure 4A).
In liver biopsies from patients with chronic hepatitis C and control patients, we found no significant differences in the mRNA expression levels of LXRα and LXRβ. However, CAR expression was significantly lower in HCV patient liver biopsies in consistence with the data in B6HCV mice (Figure 4B). Biopsies of patients are systematically taken after overnight fasting, and glucose levels are normal (< 7mmol/l) in non-diabetic patients.

Figure 4

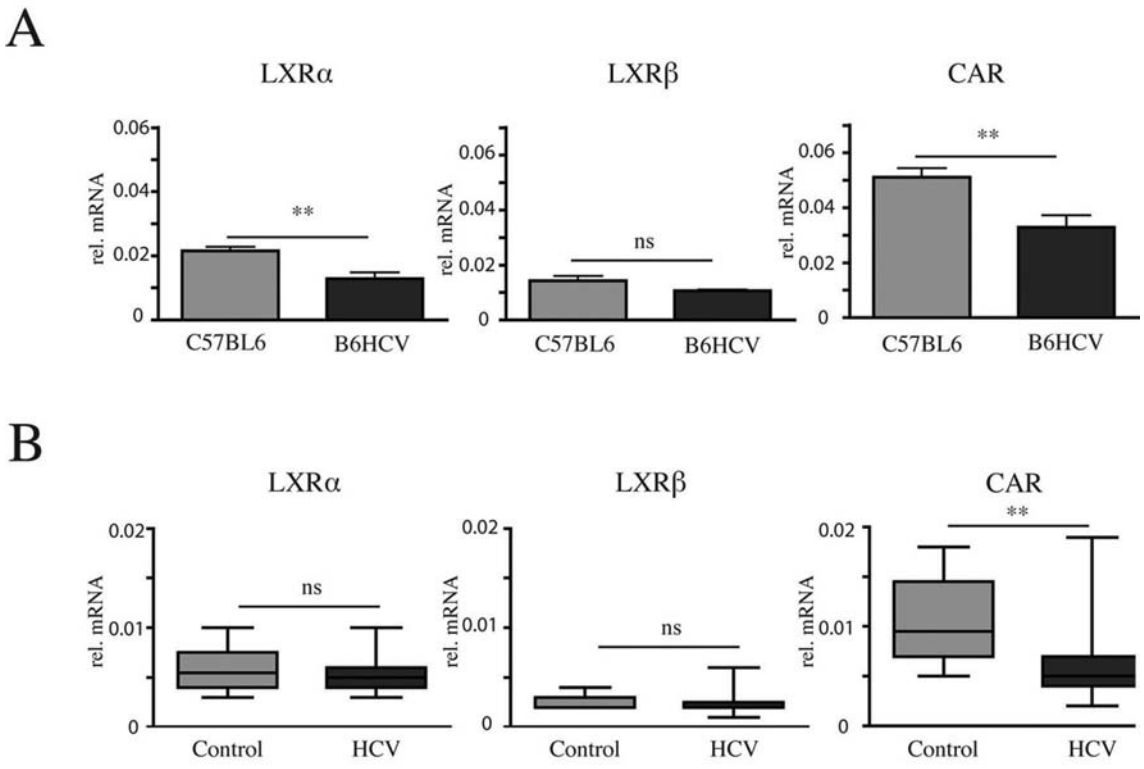

Figure 4: Expression of LXRα, LXRβ and CAR in B6HCV mice and human liver biopsies.
A) *Expression of LXRα and CAR but not LXRβ is decreased in B6HCV mice*. C57BL6 and B6HCV mice were starved for 8 hours (n=6 of each group). RNA was extracted from liver homogenates and mRNA expression of LXRα, LXRβ and CAR was assessed by quantitative RT-PCR. mRNA expression is shown as the number of copies relative to GAPDH. Statistical analysis was done using a two-tailed unpaired t-test (**, p=<0.01).
B) *Expression of CAR but not LXR is lower in human liver biopsies from HCV patients.* RNA was extracted from human liver biopsy samples of control (n=10) and HCV (n= 28) patients. Expression of LXRα, LXRβ and CAR mRNA was analysed by qRT-PCR and data was expressed as described in 4A. Statistical analysis was done using a two-tailed Mann Whitney test (**, p=<0.01).

3. *Increase in gluconeogenesis as the key mechanism of HCV induced insulin resistance by PP2A ?*

PP2Ac overexpression increases gluconeogenic gene expression in cell culture

From our experiments in cell culture we know that regulation of glucose homeostasis by insulin signaling, AMPK signaling and probably also LXR is inhibited when PP2A is overexpressed. These 3 pathways have one common endpoint: inhibition of gluconeogenesis. We therefore questioned whether inappropriate inhibition of gluconeogenesis is the key mechanism of HCV induced insulin resistance related to hepatic overexpression of PP2A.
To answer this question we studied gene expression of PGC1α, the main transcriptional coactivator for gluconeogenic genes, and PEPCK and G6Pase mRNA expression in Huh7 and HA-PP2Ac cells. We studied gene expression under low (5mM) and high (25mM) glucose conditions.
At least under high glucose conditions, expression of PGC1α, PEPCK and G6Pase was upregulated in HA-PP2A cells, implicating increased glucose production in cells overexpressing PP2A (Figure 5A).
On the other hand, when we inhibited PP2A activity in Huh7 cells by stimulation with low dose okadaic acid overnight, PGC1α, PEPCK and G6Pase were downregulated (Figure 5B). Interestingly, when we decreased PP2Ac expression by silencing with shRNA against PP2Acα, PGC1α was downregulated but not PEPCK and G6Pase (Figure 5C).

Figure 5

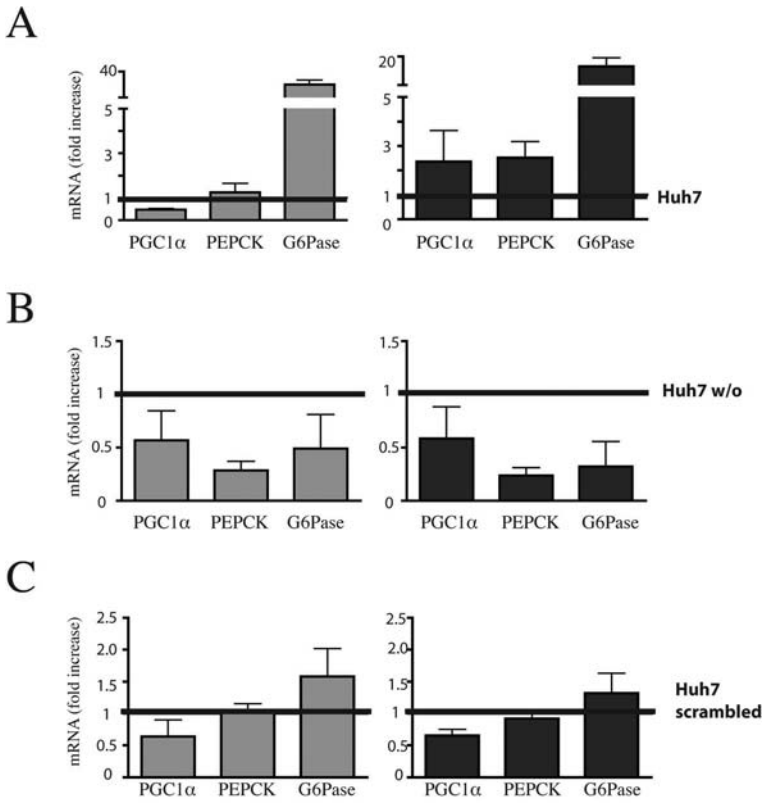

Figure 5: PP2Ac expression upregulates PGC1α, PEPCK and G6Pase in cell culture.
A) Huh7 and HA-PP2A cells were grown in low (5mM, left) and high glucose medium (25mM, right), respectively. Expression of PGC1α, PEPCK and G6Pase mRNA was analysed by qRT-PCR. Data from 3 independent experiments is shown as fold increase of gene expression in HA-PP2A cells compared to Huh7.
B) Huh7 cells were grown in low (left) and high (right) glucose medium and treated +/- Okadaic Acid (OA) 25nM o/n. Expression of PGC1α, PEPCK and G6Pase was analysed by qRT-PCR. Data from 3 independent experiments is shown as fold increase of gene expression in OA treated cells compared to non-treated Huh7.
C) Huh7 cells transfected with a shRNA vector against PP2Acα and scrambled shRNA were grown in low (left) and high (right) glucose medium. Gene expression was analysed by qRT-PCR and shown as fold increase in gene expression in PP2Acα silenced cells compared to cells transfected with scrambled shRNA.

B6HCV mice have an insulin resistant phenotype

In B6HCV mice, development of steatosis has been described but until present it is not known whether they develop insulin resistance. We have shown that insulin signaling is inhibited and AMPK is hypophosphorylated in B6HCV mice (section 4.1). To find out whether inhibition of these metabolic signaling pathways has any physiologic consequence, we analysed glucose homeostasis in these mice.

Fasting insulin and glucose were measured and HOMA-IR was calculated. Interestingly both fasting glucose and insulin were significantly higher in B6HCV mice. Therefore HOMA index, a parameter of peripheral insulin resistance, is significantly higher in B6HCV mice (Figure 6A).
A glucose tolerance test in B6HCV mice showed a higher increase in blood glucose 15 min. after glucose administration but decline was similar to C57BL6 mice. The insulin tolerance test shows, that insulin administration still induces glucose uptake into the muscle in B6HCV mice, but glucose levels remained higher at all time points in B6HCV mice (Figure 6B).
Taken together B6HCV mice have an insulin resistant phenotype.

Figure 6

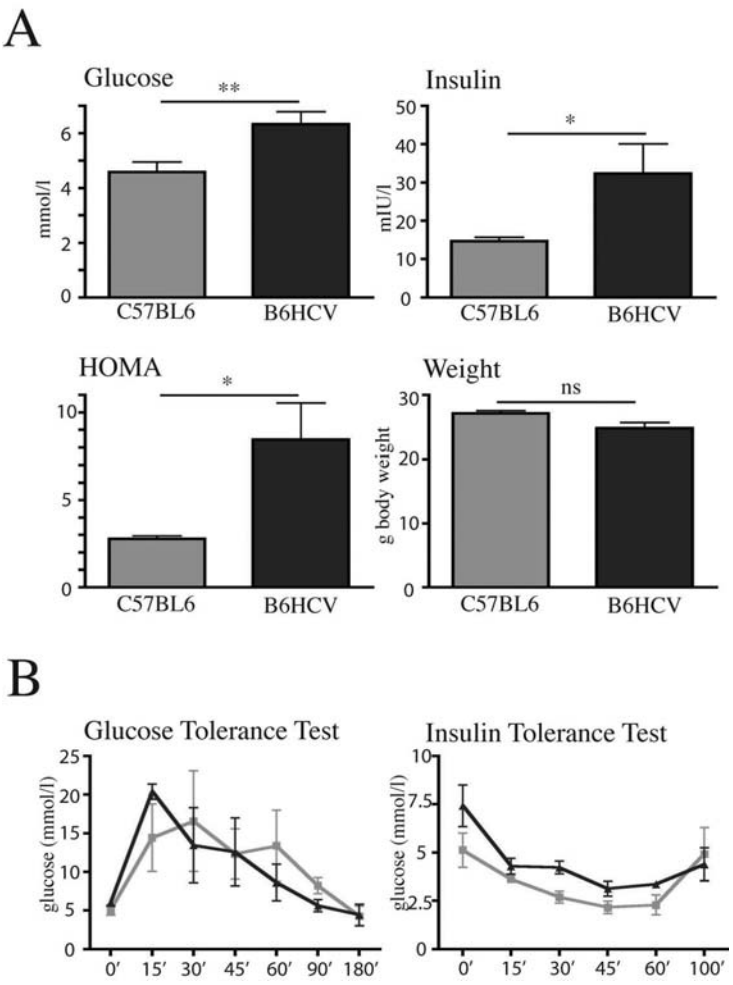

Figure 6: B6HCV mice have an insulin resistant phenotype.
A. Mice were starved o/n and fasting glucose and insulin levels were measured from tail vein blood (n=6 of each group). Glucose values are shown in mmol/l, insulin levels in mIU/l. HOMA-IR was calculated (fasting glucose x fasting insulin /22.5). Glucose (p=0.0098), insulin (p=0.047) and HOMA (p=0.0224) are significantly higher in B6HCV mice using a two-tailed unpaired t-test. Weight (in g body weight) did not differ significantly.
B. Glucose (GTT) – and insulin tolerance tests (ITT) were done in 3 mice of each group. Glucose (dose: 2 mg/kg body weight) and insulin (dose: 0.8 IU/kg body weight) were administered i.p. and glucose was measured from tail vein blood at given time points. Data is shown as mean values +/- SEM. C57BL6 grey line, B6HCV black line.

Gluconeogenic gene expression in B6HCV mice

To investigate further the reason for insulin resistance and high fasting glucose values in B6HCV mice, we measured mRNA expression of PGC1α, PEPCK and G6Pase by quantitative RT-PCR under fasting conditions.
PGC1α was significantly upregulated in B6HCV mice. Surprisingly and contradictory to our results in vitro, there was no significant difference in expression levels of PEPCK and G6Pase (Figure 7).

Figure 7

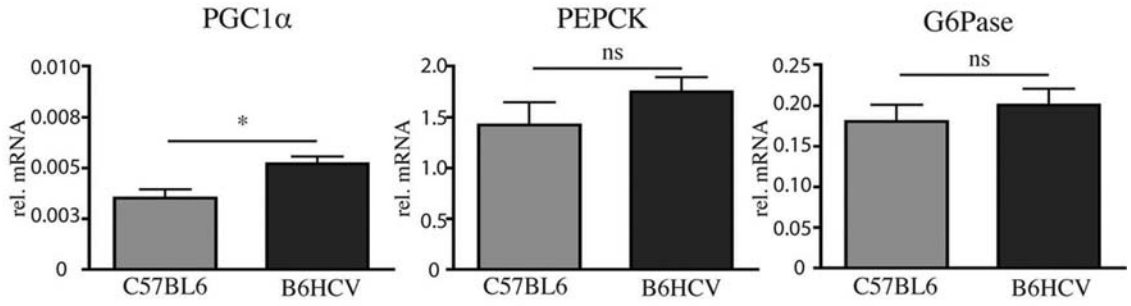

Figure 7: Transcriptional regulation of gluconeogenic enzymes in B6HCV and control mice. Mice were fasted for 8 hours (n=6 of each group). RNA was extracted from snap frozen liver tissue and transcribed to c-DNA. Relative mRNA expression was analysed using quantitative RT-PCR and shown as the number of copies relative to GAPDH. Statistical analysis was done using a two-tailed unpaired t-test (*, $p<0.05$).

Gluconeogenic gene expression in patients with chronic hepatitis C

We further analysed expression of gluconeogenic genes in 28 liver biopsies from HCV infected patients and 10 biopsies from healthy controls. Naturally patients were starved overnight at the time point of liver biopsy and had normal blood glucose levels.
In accordance with the findings in B6HCV mice, we found a significant upregulation of PGC1α but not PEPCK or G6Pase mRNA expression in HCV patients (Figure 8). Interestingly we also found that GLUT2, the major glucose transporter in the liver, is expressed at lower levels in the HCV group, suggesting lower hepatic glucose uptake.

Figure 8

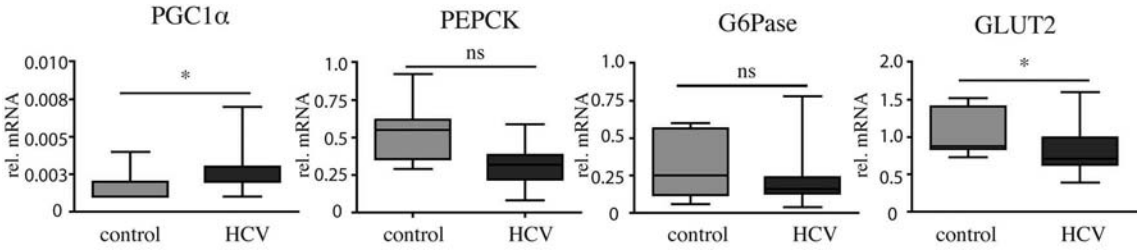

Figure 8: Transcriptional regulation of gluconeogenic enzymes in HCV patients
A small part of the liver biopsy samples from 28 HCV and 10 control patients was stabilized in RNA later and frozen. RNA was extracted and transcribed to c-DNA. Relative mRNA expression was analysed using quantitative RT-PCR and shown as the number of copies relative to GAPDH. Statistical analysis was done using two-tailed Mann Whitney Test (*, $p<0.05$).

4. *Insulin Resistance and natural history of NAFLD and NASH – first data from the NAFLD Cohort study in Basel*

Patient characteristics of the Basel NAFLD cohort study population

Since March 2003 we have included 57 patients with histological diagnosis of NAFLD (22 patients, 39%) or NASH (35 patients, 61%) into the cohort study. Follow up of patients is done once per year and during this time period, 40 patients have completed follow up visit 1, 24 completed follow up visit 2, 17 completed follow up visit 3 and 8 completed follow up visit 4. 11 patients stopped study participation for different reasons. We included 37 male patients and 20 female patients whereas mean age at entry was 44 years for male and 50 years for female patients.
Descriptive statistics given here are calculated from the data collected at baseline visits of the 57 patients included. Longitudinal observations cannot be calculated yet as follow up of patients over a defined period has not been completed.
Analysis of the liver histology shows that most of the patients have severe steatosis. Steatosis was staged according to the NAS scoring system (139). 18% of patients had only mild steatosis (5-33% of surface area of hepatic parenchyma), 28% had moderate steatosis (34-66%) and 54% of patients had severe steatosis ($\geq$ 67%). Within the NASH group, 7 patients (12% of all patients) present histological features of cirrhosis at the date of liver biopsy.
NAFLD has been referred to as the liver manifestation of the metabolic syndrome. Metabolic syndrome according the criteria of the International Diabetes Federation (IDF) (140) was diagnosed in 51% of patients at baseline. Preliminary data shows that another 7.5% developed metabolic syndrome during the observation period. Most of the patients had overweight or adiposity while only 14% had normal BMI (BMI <25 kg/m^2) and 12% had normal waist circumference (<94 cm for male and <80 cm for female patients).
Diabetes was present in 18% of patients at baseline. Preliminary data shows that another 10% developed diabetes during follow up. On the other hand, insulin resistance measured by HOMA-IR, a major pathogenic factor of both metabolic syndrome and NAFLD, was seen in 78% of patients. Also dyslipidemia, defined as hypertriglyceridemia or hypercholesterolemia, was frequently observed in our study population (72%). Hypertension was seen in 35% of patients and only 4% had coronary heart disease.

Patients with NASH have significantly higher BMI and insulin resistance

One of the major questions in NAFLD is to find a non-invasive diagnostic tool to distinguish patients at risk for NASH from patients with uncomplicated NAFLD.
To screen for discrepancies in clinical parameters and laboratory values between NAFLD and NASH patients, we analysed the mean values of different parameters for each of the 57 patients included into our NAFLD cohort study and followed from March 2003 until January 2008. Differences were calculated using the Mann Whitney test.
A clinical risk factor for the development of NAFLD is adiposity. It can be assessed by the body weight or the body mass index (BMI). Central adiposity, a consequence of visceral fat accumulation, has been considered worse than peripheral adiposity, a consequence of subcutaneous fat accumulation. Waist circumference is a parameter that roughly estimates the

amount of visceral fat. In our population we found that BMI (p=0.0465) and waist circumference (p=0.003), but not body weight alone (p=0.2380), are significantly higher in the NASH group (Figure 9A).
We further found that insulin resistance, calculated by HOMA-IR, an important factor in the pathogenesis of NAFLD, is significantly higher in NASH than in NAFLD patients (p= 0.0019). Also fasting glucose (p=0.02) and insulin (p=0.0011) are significantly higher in the NASH group (Figure 9B).
In clinical practice, liver aspartate aminotransferase (AST), alanine aminotransferase (ALT) and γ-glutamyl transpeptidase (GGT) are measured in the blood in order to estimate the activity of hepatic inflammation. The ratio AST/ALT has been proposed to predict NASH in NAFLD (141). When comparing these hepatic enzymes in fasting blood samples we observe significantly higher values for AST (p=0.0122) but not ALT (p=0.3986) and GGT (p=0.1402) values (Figure 9C).
In a multivariate regression analysis, HOMA-IR (p=0.01), fasting glucose (p=0.01) and fasting insulin (p=0.001) were significantly correlated with the diagnosis of NASH. Also AST (p=0.002) but not GGT (p=0.44) has been associated with NASH. BMI (p=0.02) also showed correlation with the diagnosis of NASH whereas weight (p=0.43) and waist circumference (p=0.12) were not associated. This correlation disappears (p=0.121) when we remove weight and waist circumference from the regression.

Figure 9

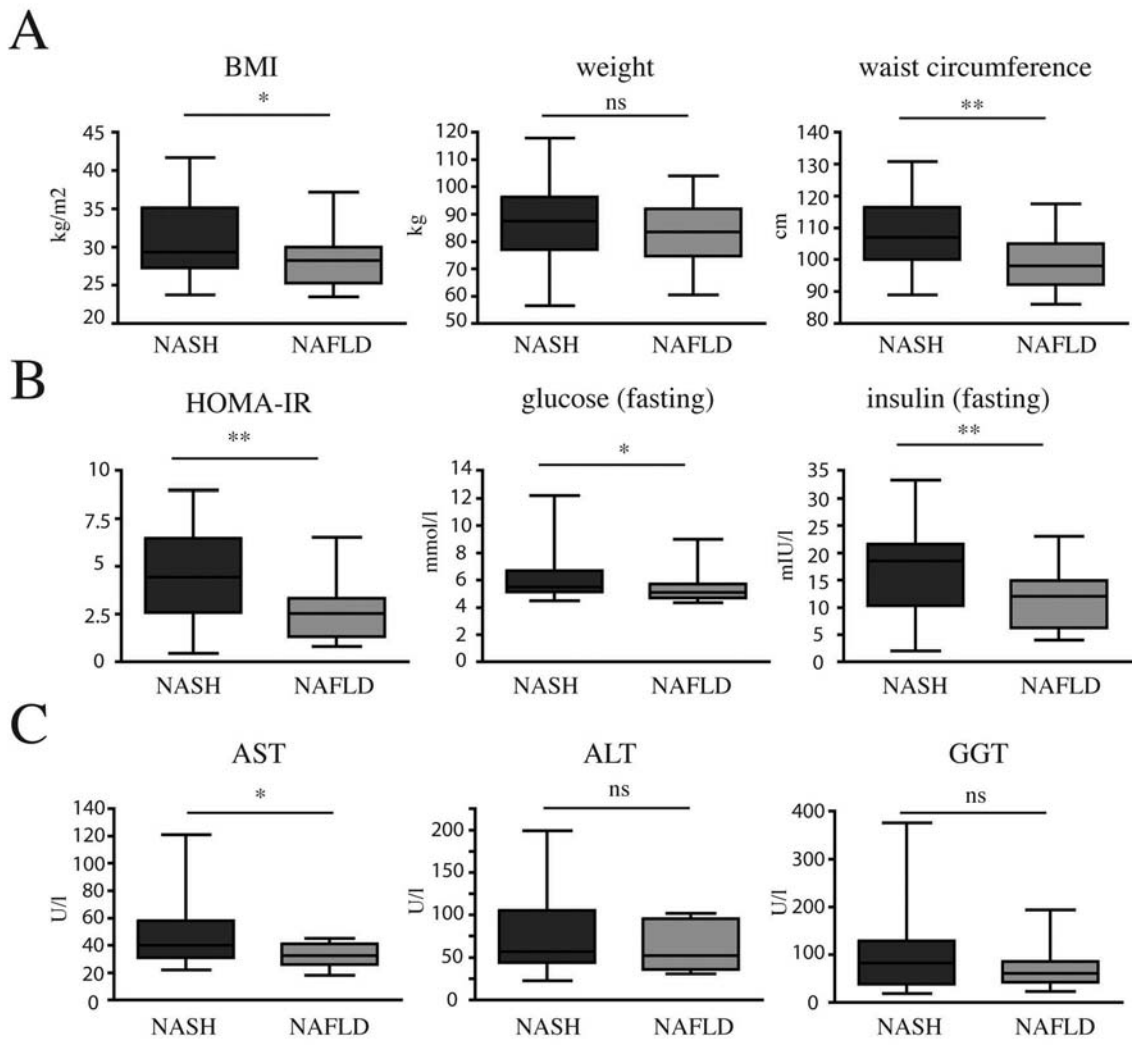

Figure 9: Differences in clinical and laboratory values between NAFLD and NASH patients. Data of 35 NASH and 22 NAFLD patients has been collected at preferentially all visits. Mean values of the different parameters were calculated for each patient and compared between the 2 groups of diagnosis by a non-parametric test (two-tailed Mann Whitney Test) (*, $p<0.05$; **, $p<0.01$).

A. Weight (kg), BMI (kg/m^2) and waist circumference (cm) were measured in all patients.

B. Fasting glucose (mmol/l) and insulin (mIU/l) were measured in blood samples, HOMA-IR was calculated.

C. AST (U/l), ALT (U/l) and GGT (U/l) were measured in fasting blood samples.

Cytokeratin-18 fragmentation is a parameter to distinguish NAFLD from NASH

It recently has been proposed that assessment of cytokeratin-18 (CK-18) fragments in the blood might be used as a non-invasive marker to predict NASH in NAFLD (42, 43). CK-18 fragmentation is believed to reflect liver cell apoptosis and therefore correlate with severity of NAFLD. CK-18 fragments have been measured in the blood of 44 patients with NAFLD and assigned to liver histology. A cutoff- value of 395 U/l of CK-18 fragments has been calculated and showed a specificity of 99.9% and a sensitivity of 87.5% to predict NASH (42).
We measured CK-18 fragments in serum samples from 31 NASH and 16 NAFLD patients at their baseline visits. The value was assigned to histological diagnosis of NAFLD or NASH. In contrast to the original paper from Wieckowska et al., we did not assign a group of patients with borderline NASH. NASH patients had significantly higher CK-18 values (Median 245.6 IU/l in NASH versus 163.4 IU/l in NAFLD, p= 0.0056, two-tailed Mann Whitney Test) (Figure 10).

Figure 10

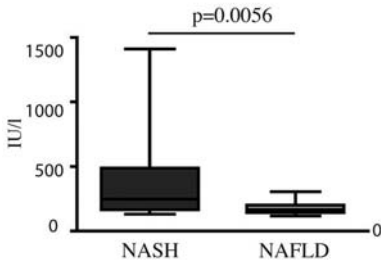

Figure 10: *NASH patients have significantly higher CK-18 fragments than NAFLD patients.* CK-18 fragments were measured in the serum of 31 NASH and 16 NAFLD patients at baseline.

5. Discussion

1. Dysregulation of hepatic glucose metabolism by protein phosphatase 2A - a new mechanism for viral induced insulin resistance development

Development of insulin resistance and type 2 diabetes mellitus involve highly complex systemic mechanisms that have not been clearly described yet.
Viral induced insulin resistance in contrast is a more restricted scenario since it is primarily limited to the organs and tissues infected by the virus. In the case of hepatitis C studied in this work, presence of viral particles in the liver is regarded as the origin of insulin resistance development.
There are at least two possibilities for the HCV virus to contribute to systemic insulin resistance:
First the viral proteins could directly or indirectly interfere with glucose metabolism in the liver and thereby contribute to hepatic insulin resistance – inappropriate glucose regulation in the liver in response to insulin. This implies inappropriate glucose uptake, glycogen storage and increased hepatic glucose production and can lead to hyperglycemia and secondary to peripheral insulin resistance.
Second the viral infection could induce cytokine production and release from activated mononuclear cells and immune competent cells in the liver. Cytokines would interfere with glucose metabolism in response to insulin in the liver and in peripheral organs leading to systemic insulin resistance.
Evidence and model systems for both possibilities have been described. For instance, indirect interference of viral proteins with the insulin signaling pathway have been reported through the upregulation of SOCS-3 (142), SOCS-7 or activation of mTOR (127) mediated degradation of IRS-1. Upregulation of the cytokines IL-6 (136, 143) and TNFα (121, 137) in response to viral infection have also been proposed to interfere with the insulin signaling cascade and initiate insulin resistance development.
Our work was initiated by the observation that protein phosphatase 2A is transcriptionally upregulated in the virus infected liver by induction of an ER stress response. Transcriptional upregulation of one of the major serine-/threonine phosphatases is a critical event as it can disturb multiple cellular signaling cascades in the liver that are controlled by serine-/ threonine phosphorylation. We have already reported inhibition of interferon signaling by PP2A (57). Researchers from a different laboratory had observed inhibition of insulin signaling by PP2A in adipocytes (131). Also ER stress has been implicated in pathogenesis of insulin resistance (65, 144). There is sufficient evidence to assume that virus induced PP2A upregulation in the liver might dysregulate signaling cascades that control glucose metabolism.
In the work presented in this thesis we were able to establish a new model for virus induced insulin resistance development. We provide evidence in vitro and in vivo that overexpression of PP2A in the liver inhibits the insulin signaling pathway, AMPK phosphorylation and probably also LXR regulated inhibition of gluconeogenesis. PP2A therefore interferes with three different mechanisms involved in the control of hepatic glucose metabolism.
Detailed analysis of the insulin signaling cascade revealed that PP2A directly or indirectly dephosphorylates and inactivates PKB/Akt, a kinase centrally involved in the pathway.

Contrary to others cited above (121, 126, 127, 142) but in accordance with Aytug et al., (130) we did not find inhibition of insulin signaling upstream of PKB/Akt. Further downstream signaling is decreased, here shown for GSK3β phosphorylation, but most likely also Foxo transcription factors, PGC1α, AS160 and other known substrates for PKB/Akt (85). Inhibition of insulin signaling at the level of PKB/Akt leads to decreased glucose uptake, decreased glycogen synthesis and impaired inhibition of gluconeogenesis contributing to hyperglycemia. We provide evidence that glycogen synthesis is impaired through hypophosphorylation of GSK3β unless there is debate about the substantial role for GSK3β in regulating glycogen synthase (145, 146). Further glucose uptake seems to be affected as we observed significantly lower expression of GLUT2 in HCV liver biopsies. We additionally observed that PGC1α, the main coactivator for gluconeogenic enzymes, is transcriptionally upregulated by PP2A in vivo and in vitro. PEPCK and G6Pase were also upregulated in vitro under high glucose conditions implying inappropriate increase of gluconeogenesis. Unexpectedly, this was not observed in vivo, neither in mouse- nor in human liver tissue. It is possible that differences in PEPCK and G6Pase cannot be observed under low glucose levels, when these enzymes are maximal expressed. Experiments in both mice and human were done in a fasted state. Further gluconeogenesis is regulated not only by transcriptional regulation of the required enzymes but also by enzyme activity in a function of the amount of substrates. To finally prove the concept that PP2A is upregulating hepatic glucose production we further need to study glucose production in the supernatant of hepatocytes that were transfected with a PP2Ac expressing construct. Additionally gene expression and enzymatic activity of PEPCK and G6Pase need to be analysed in B6HCV mice in the fed state.

Gluconeogenesis is tightly controlled by AMP kinase that can be activated by metformin , a widely used and effective anti-diabetic drug. We show in cell culture and in HCV mice that PP2A dephosphorylates AMPK, thereby inhibiting its activity and its ability to suppress gluconeogenesis. Unfortunately, it is not feasible to study AMPK phosphorylation in human liver biopsies under routine conditions, because AMPK is phosphorylated due to ATP depletion within few minutes after obtaining the biopsy. These data provide evidence for PP2A dependent increase of PGC1α expression and gluconeogenesis. Regulation of AMPK by PP2A has been described in rat hepatocytes (104) and β-cells (105). Supporting our model of PP2A as an important inhibitor of AMPK and other pathways of glucose metabolism, it has recently been published that palmitate, a saturated free fatty acid (FFA), would activate PP2A and hereby inhibit AMPK phosphorylation (106). FFAs in the circulating blood are increased in the insulin resistant state and play an important role in the maintenance of insulin resistance and fatty liver disease. As described, two different models have been published to clarify the mechanism by which metformin phosphorylates and activates AMPK (102, 103). It would be interesting to know whether metformin interferes with PP2A activity.

Besides insulin and AMP/ATP levels, hepatic gluconeogenesis can be suppressed by glucose itself. We show that PP2A overexpression impairs glucose induced gene regulation in vitro by a yet unknown mechanism. Recently LXR have been implicated in glucose induced regulation of glucose- and lipid-metabolism (110). Glucose has an effect on LXR subcellular localization in β-cells. High glucose conditions lead to nuclear translocation of LXR, probably regulated by LXR phosphorylation (116). Little is known about the role of LXR phosphorylation on serine 198 (115) but it might regulate its coactivator- or corepressor recruitment thereby changing its transcriptional activity (117). Changes in transcriptional activity due to phosphorylation have been described for other nuclear receptors, among them CAR, that is regulated by PP2A (119, 120). We speculate that PP2A dephosphorylates LXR. If LXR serine phosphorylation indeed changes transcriptional activity and glucose is a ligand

at LXR, this could explain why PP2A impairs glucose induced regulation of gluconeogenic genes.
We propose a model here where virus induced upregulation of PP2A acts in a key position and interferes with three independently regulated pathways that control gluconeogenesis. Furthermore it negatively regulates glucose uptake and glycogensynthesis.

2. Systemic insulin resistance and ER stress as a drug target

It is of great interest to elucidate mechanisms of virus induced insulin resistance because insulin resistance itself might contribute to complications such as progression of hepatic fibrosis and resistance to antiviral drugs. Additional drugs counteracting insulin resistance development could help to avoid progressive forms of chronic hepatitis C and to improve response to antiviral therapy.
Having pointed out a new mechanism of HCV interference with hepatic signaling pathways in glucose metabolism, we investigated its significance in systemic insulin resistance development in vivo.
Little is known about the phenotype of the B6HCV transgenic mice studied here. It has been observed that up to 80% of animals develop steatosis and 60% show different patterns of inflammatory infiltration when they are older (124). Whether mice develop insulin resistance prior to steatosis and whether fibrosis or hepatocellular cancer formation occurs as a consequence of chronic inflammation has not been described. Another HCV genotype 1b transgenic mouse has been shown to develop insulin resistance and also diabetes, when fed with a high fat diet (121).
We show for the first time that B6HCV mice have an insulin resistant phenotype with elevated levels of fasting glucose, fasting insulin and lower insulin tolerance at 3 months of age. From our gene expression analysis, we propose that PP2A dysregulates mainly gluconeogenesis through overexpression of PGC1α and leads to increased hepatic glucose production. To confirm this hypothesis, gluconeogenesis in B6HCV mice might be assessed by a radioactive assay measuring the amount of newly synthesized glucose from a C-14 labeled alanine substrate.
Having detected development of insulin resistance, we are further interested in the phenotype of B6HCV mice. As insulin resistance in HCV is associated with diverse consequences, we would like to study development of steatosis, steatohepatitis, fibrosis and eventually HCC in dependance of fasting glucose and –insulin. As described, free fatty acids, which are relaesed from the adipose tissue in the insulin resistant state, probably play a prominent role in the development of type 2 diabetes but also steatosis. We plan to feed B6HCV mice with high fat diets (HFD) consisting of different fat sources – saturated and unsaturated fatty acids. We expect accelerated development of steatosis and eventually steatohepatitis and fibrosis compared to mice on a regular diet. Further we would expect development of overt diabetes in mice on HFD, especially in the saturated free fatty acid group. It has been shown recently that palmitic acid, a saturated free fatty acid, acitvates PP2A and inhibits AMPK phosphorylation whereas oleic acid, a monounsaturated free fatty acid, did not (106). In our mouse model, where hepatic PP2A expression is increased, the different free fatty acid composition might influence the insulin resistant state and histologic findings.
In human HCV liver biopsies we show that PP2A is upregulted and inhibits insulin signaling. Gene regulation analysis shows an upregulation of gluconeogenesis by PGC1α and decreased glucose uptake by GLUT2. Our data hint at a phenotype of hepatic insulin resistance. From the literature we know that HCV patients have elevated HOMA levels, a parameter for

systemic insulin resistance (14-18). We propose a model where PP2A induces hepatic insulin resistance leading to increased hepatic glucose production and decreased uptake. Hyperglycemia then might contribute to systemic insulin resistance with involvement of adipose tissue and muscle and development of compensatory hyperinsulinemia that is assessed by the HOMA-IR. In a large number of patients we did not observe a correlation between hepatic PP2A expression or the phosphorylation levels of PKB/Akt with HOMA-IR, supporting a two step model leading to systemic insulin resistance in chronic heapatitis C.
The mechanism developed here is caused by virus induced ER stress (70). ER stress is increased in the liver under diabetic conditions (65, 147, 148). A drug preventing ER stress would eventually prevent the diverse consequences on hepatic and systemic insulin resistance. Chemical chaperones such as 4-phenyl butyric acid (4-PBA), trimethylamine N-oxide dihydrate (TMAO) and dimethyl sulfoxide are low molecular weight compounds that stabilize protein conformation, improve protein folding und trafficking (64). Further bile acid derivates as tauroursodeoxycholic acid (TUDCA) can modulate ER function (149). It has been described in a mouse model of type 2 diabetes mellitus (leptin deficient ob/ob mice) that 4-PBA and TUDCA can restore glucose homeostasis by the prevention of ER stress (66). UDCA is a drug widely used in cholestatic liver disease and has no severe side effects. Its use in the treatment of steatohepatitis is discussed controversely, while a large study in 2004 did not find benefit for UDCA compared to placebo (150), one recent study shows reduction in ALT values and in hepatic steatosis by UDCA (151). In Hepatitis C, UDCA also lead to decrease in ALT values but its ability to prevent disease progression has not been shown yet (152, 153). One the other hand it has been shown that bile acids can promote HCV replication in cells (154) disencouraging their use in chronic hepatitis C. Chemical chaperones have not been tested in clinical trials yet.
To evaluate the effect of ER stress preventing agents as drugs for insulin resistance in hepatitis C, we consider feeding B6HCV mice with TUDCA or 4-PBA and study the effect on hepatic insulin signaling and systemic insulin sensitivity. If the experiments show a beneficial effect of these agents, they might be used as preventive drugs in chronic HCV infection.

3. Insulin resistance and apoptosis in the progression of NAFLD

Since introduction of the historical “two hit hypothesis” for pathogenesis of NAFLD it has been stressed repeatedly that insulin resistance plays a prominent role. Sequential resistance of peripheral tissues and the liver to insulin has been hypothesized (134). Hereby including the role of increasing free fatty acids from the insufficient suppression of lipolysis and increased FFA production in the insulin resistant state. However the precise mechanisms are still unknown. Cell injury may occur when the capacity of cells to safely store fat is overextended and fatty acids become toxic to the cell and induce so called “lipotoxicity”, apoptosis induced by fatty acids (155).
Preliminary multivariate regression analysis of our cohort of NAFLD and NASH patients shows that NASH is independently associated with insulin resistance. This result confirms a key role for insulin resistance in the pathogenesis of NASH.
Cytokeratin-18 fragments in the blood have been proposed to predict NASH, as a non-invasive marker for liver cell apoptosis (42, 43). In accordance with this, analysis of CK-18 fragmentation in our cohort reveals that NASH patients have significantly higher CK-18 fragments.

Searching for a parameter to predict NASH, also insulin resistance by HOMA might be a potential candidate. Further AST is independently associated with NASH whereas GGT and ALT are not. An AST/ALT or AST/GGT ratio might therefore be good parameters. AST/ALT ratio >1 has already been proposed to predict advanced fibrosis in NAFLD (141).
We furthermore propose that measurement of CK-18 fragments by ELISA should be established as a routine test and would be useful in combination with the other clinical parameters discussed above. However, these methods can so far not replace the histological assessment of the diagnosis.
It is not clear whether BMI has a significant influence on NASH but both body weight alone and waist circumference were not related to the diagnosis. The fact that waist circumference is not related to NASH queries the current model of waist-to-hip ratio. It is believed that visceral fat, roughly estimated by measuring the waist circumference, is more harmful than subcutaneous fat. In the insulin resistant state, FFA from visceral fat but not the subcutaneous fat would directly enter the liver by the portal vein. We did not observe any correlation between the waist circumference and NASH and should therefore reconsider the method to estimate visceral fat and the current hypothesis described above.
Until present we do not have the collective for longitudinal analysis of these parameters. The development of HOMA, insulin and glucose, respectively, but also CK-18 fragments over time and dependent on the histological diagnosis will be interesting to observe. We further would like to study the predictive value of these parameters by comparison to a second liver biopsy 5 to 10 years after the initial biopsy. Correlation of development in both histology and serum markers could define non-invasive methods to distinguish NAFLD from NASH.

6. Summary

The work presented here analyses the role of PP2A in the dysregulation of glucose metabolism in the setting of chronic hepatitis C. We propose a new model for virus induced insulin resistance development through the overexpression of PP2A in the liver that leads to hyperglycemia mainly through the inappropriate inhibition of hepatic gluconeogenesis.
By dephosphorylation of key targets, PP2A upregulation interferes with three differently regulated pathways that control hepatic glucose metabolism schematically shown in figure 11. First, it interferes with the insulin signaling cascade (right) by dephosphorylation of PKB/Akt, thereby upregulating gluconeogenesis and inhibiting glycogensynthesis. Second, it dephosphorylates AMPK and therefore again increases gluconeogenesis (center). And finally glucose induced regulation of gluconeogenesis is impaired through PP2A upregulation and eventually involves regulation of LXR (left).
This concept has been developed in cell culture, but in a model of HCV transgenic mice we show that hepatic PP2A overexpression and dysregulation of these signaling pathways presents concomitantly with an insulin resistant phenotype. Similary, in patients infected with chronic hepatitis C we observed upregulation of PP2A and inhibition of hepatic insulin signaling, supporting its significance in vivo.
A model explaining HCV induced insulin resistance development can help to understand disease progression to liver cirrhosis and resistance to antiviral therapy. It further discloses new drug targets in order to prevent these complications.

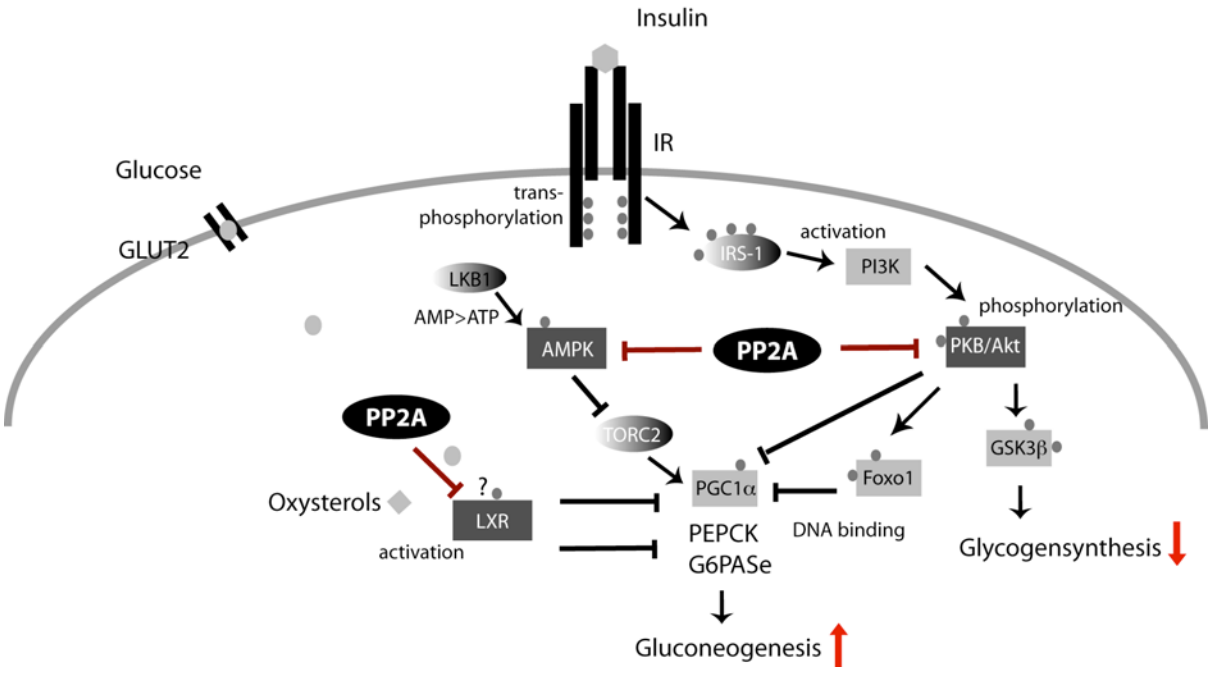

Figure 11: *Model for PP2A interference with glucose regulating pathways in the liver*

Concerning the clinical course of NAFLD and NASH, respectively, preliminary data from our observational study support the importance of insulin resistance and liver cell apoptosis in the pathophysiology of disease progression. We further confirm the use of CK-18 fragmentation in the blood as a non-invasive marker predicting NASH.

7. References

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