Exploring anti-fibrotic drugs
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Chapter B1

Pathophysiological model of non-alcoholic fatty liver disease using precision-cut liver slices

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Abstract

**Background and purpose**
Current *in vitro* non-alcoholic fatty liver disease (NAFLD) models fail to capture the essential interactions between liver cell-types and do not reflect the pathophysiological status of NAFLD patients. Precision-cut liver slices (PCLS) are an effective *ex vivo* model to study multicellular diseases, since the interplay between liver cell-types is maintained. Therefore, this study aimed to develop PCLS to study NAFLD.

**Experimental approach**
Steatosis was induced by culturing rat PCLS in modified culture media which contained pathophysiological serum concentrations of glucose, fructose, insulin and palmitic acid as seen in NAFLD patients, for up to 48h. The steatosis was evaluated using Oil Red O staining. Furthermore, expression of carbohydrate/lipid metabolism-related genes was assessed by quantitative real-time PCR.

**Key results**
Liver steatosis, characterized by intracellular microvesicular lipid droplets, was successfully induced in PCLS cultured in medium containing insulin. The microvesicular steatosis was in line with the down-regulation of genes encoding carnitine palmitoyltransferase 1 (Cpt1) which plays a role in fatty acid transport and mitochondrial β-oxidation. On the other hand, the up-regulation of markers of de novo lipogenesis: acetyl-CoA carboxylase 1 (Acc1), carbohydrate responsive element binding protein (Chrebp) and sterol regulatory element binding protein 1c (Srebp-1c), was not always paralleled by steatosis observed in the PCLS.

**Conclusion and implications**
Steatosis can be induced in PCLS using modified culture media, which reflects the pathophysiological development of NAFLD. The disruption of fatty acid transport and mitochondrial β-oxidation are probably the main pathways responsible for the accumulation of microvesicular steatosis in our PCLS model. This novel pathophysiological *ex vivo* model can be used as an effective tool to study NAFLD-associated conditions and test the efficacy of anti-NALFD drugs.

**Key words**: NAFLD; non-alcoholic fatty liver disease; *ex vivo*; pathophysiological model.

**Abbreviations**: NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; VLDL, very low-density lipoprotein; PCLS, precision-cut liver slices; BSA, bovine serum albumin; Ywhaz, tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein, zeta; Acc, acetyl-CoA carboxylase; Srebp, sterol regulatory element binding protein; Chrebp, carbohydrate responsive element binding protein; Cpt, carnitine palmitoyltransferase.
Introduction

Non-alcoholic fatty liver disease (NAFLD), or hepatic steatosis, is characterized by excessive deposition of lipids in the liver in the absence of excessive alcohol intake [1, 2]. Although NAFLD, the most common liver disorder worldwide, may be benign, chronic and unresolved liver steatosis can progress to non-alcoholic steatohepatitis (NASH), cirrhosis and even hepatocellular carcinoma [1-3]. Furthermore, the clinical burden of NAFLD is not only confined to liver-related problems, uncontrolled NAFLD can aggravate extra-hepatic diseases such as diabetes mellitus, cardiovascular disease and chronic kidney disease [4]. To date, no NAFLD- or NASH-specific pharmacological interventions are clinically approved, and the only well-established option for the management of NAFLD are life-style modifications [5, 6].

The biggest risk factors for NAFLD are metabolic disorders, in particular hyperglycemia and insulin resistance [7-11]. Chronic hyperglycemia and hyperinsulinemia disrupt the systemic physiological regulation of carbohydrate/lipid metabolism leading to the development of NAFLD [10-12]. Recently, several studies have shown that fructose also plays a role in the pathogenesis of hepatic steatosis [13-15]. Excessive intake of fructose alters carbohydrate/lipid metabolism in the liver, adipose tissue, gastrointestinal tract and nervous system [16]. In the liver, fructose activates de novo lipogenesis and increases very low-density lipoprotein (VLDL) synthesis, which ultimately contributes to the accumulation of lipid droplets [15]. Besides dysglycemia, dyslipidemia is an important risk factor in the development of NAFLD [9]. Fatty acids directly promote lipotoxicity and play a role in the progression to NASH [17, 18]. The lipotoxicity of fatty acids was frequently illustrated in vitro by using palmitic acid, the most abundant saturated fatty acid in the human body [19-21]. Palmitic acid is converted to palmitoyl-CoA in the cytosol before entering the mitochondrial matrix, via the L-carnitine shuttle system, where it undergoes β-oxidation [18]. In addition, disruption of mitochondrial β-oxidation is key in the progression of NAFLD [11, 22].

Currently, several in vitro models are used to study the pathogenesis of NAFLD [23, 24]; however, most of them fail to capture the essential interactions between hepatocytes and other cell-types such as hepatic stellate cells and Kupffer cells. In addition, in vitro and animal models often do not reflect the pathophysiological status of NAFLD patients [11, 22, 25]. Precision-cut liver slices (PCLS) have been proven to be an ideal ex vivo model to study multicellular diseases, since the interplay between the various liver cell-types is maintained [26, 27]. Therefore, we aimed to develop an ex vivo NAFLD model using PCLS, by mimicking pathophysiological risks factors in NAFLD patients.

Methods

Animals

Male Wistar rats, 12-16 weeks old, were purchased from Charles River (Sulzfeld, Germany). The experiments were approved by the Animal Ethical Committee of the University of Groningen.
**Precision-cut liver slices (PCLS)**

PCLS, with an estimated thickness of 250-300 µm, were prepared and cultured under continuous supply of 80% O\textsubscript{2}/5% CO\textsubscript{2} up to 48h, as previously described [27]. Culture media was refreshed every 24h.

**Modification of culture medium**

Williams medium E with Glutamax (Invitrogen, Bleiswijk, the Netherlands) supplemented with gentamycin (50 mg/mL; Invitrogen) was used for PCLS culture. Five different culture media were prepared to mimic the pathophysiological concentrations of glucose (Merck, Darmstadt, Germany), fructose (Merck), human insulin (Sigma, St. Louis, US) and palmitic acid (Sigma) in the serum of NALFD patients (Table 1).

**Table 1: Modification of culture medium**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Characteristic</th>
<th>Glucose</th>
<th>Fructose</th>
<th>Insulin</th>
<th>Palmitic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>Unmodified</td>
<td>11.1 mM</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M2</td>
<td>Additional glucose</td>
<td>25 mM</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M3</td>
<td>Additional glucose and fructose</td>
<td>25 mM</td>
<td>5 mM</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M4</td>
<td>Additional glucose, fructose and insulin</td>
<td>25 mM</td>
<td>5 mM</td>
<td>1 nM</td>
<td>-</td>
</tr>
<tr>
<td>M5</td>
<td>Additional glucose, fructose, insulin and palmitic acid</td>
<td>25 mM</td>
<td>5 mM</td>
<td>1 nM</td>
<td>240 µM</td>
</tr>
</tbody>
</table>

Palmitic acid solutions were prepared via complexation of palmitic acid with bovine serum albumin (BSA; Sigma). Briefly, palmitic acid was dissolved in 0.1 M sodium hydroxide (Merck) at 70 °C and subsequently mixed with preheated BSA solution at 55 °C. In addition, BSA solution without palmitic acid was added into M1-M4 to mimic the concentration of BSA (0.04%) in M5.

**Oil Red O lipid staining**

Steatosis was evaluated by Oil Red O staining. Briefly, 4 µm cryosections of PCLS were fixed with 4% formaldehyde/PBS for 10 minutes before staining with Oil Red O solution (0.6% Oil Red O in 36% 2-propanol) for 10 minutes at room temperature. The sections were counterstained with hematoxylin and examined using light microscopy. Three PCLS from each treatment-group were used per experiment. The fat content (fat-ratio) was determined by measuring the amount of lipid droplets (red area) per nucleus (blue area) using ImageJ software (US National Institutes of Health).
Quantitative real-time PCR

Expression of key genes involved in carbohydrate/lipid metabolism was assessed by quantitative real-time PCR. From each experiment, three PCLS were pooled, snap frozen in liquid nitrogen and stored at -80 °C until analysis. Total RNA was isolated using the RNeasy Lipid Tissue Mini Kit (Qiagen, Venlo, the Netherlands). Reverse transcription was performed using the Reverse Transcription System (Promega, Leiden, the Netherlands). Gene expression was determined using specific primers (Table 2) and the SensiMix™ SYBR Hi-ROX kit (Bioline, Luckenwalde, Germany). Quantitative real-time PCR was performed using a 7900HT Real Time PCR apparatus (Applied Biosystems, Bleiswijk, the Netherlands) with 1 cycle at 95 °C/10 minutes followed by 45 cycles of 95 °C/15 seconds, 60 °C/30 seconds and 72 °C/30 seconds. Ct values were corrected for the Ywhaz (ΔCt) and compared to medium M1 (ΔΔCt). Results are displayed as fold induction (2^-ΔCt).

Table 2: Primer sequences used in quantitative real-time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acc1 (Acetyl-CoA carboxylase 1)</td>
<td>GCCATCCGTTGGTTGTCA</td>
<td>GGATACCTGAGTTTGGAGCCCA</td>
</tr>
<tr>
<td>Acc2 (Acetyl-CoA carboxylase 2)</td>
<td>AATTTGTCAACCGCTTGGGA</td>
<td>CATCACACTTGACCGACCGATA</td>
</tr>
<tr>
<td>Srebp-1a (Sterol regulatory element binding protein 1a)</td>
<td>GAGCTACCTTCGGTGAGG</td>
<td>CAAATAGCCAGGGAGACTGCA</td>
</tr>
<tr>
<td>Srebp-1c (Sterol regulatory element binding protein 1c)</td>
<td>GGAGCCATGGATTCACATT</td>
<td>AGGCCAGGGAGACTGCA</td>
</tr>
<tr>
<td>Chreb (Carbohydrate responsive element binding protein)</td>
<td>GAAGACCAGGAGACCATGTC</td>
<td>TCTGACAAACAGCAGGAGGT</td>
</tr>
<tr>
<td>Cpt1 (Carnitine palmitoyltransferase 1)</td>
<td>TCTTGCAGTCAGTCACCTT</td>
<td>TCCACAGCAGACGACGAC</td>
</tr>
<tr>
<td>Ywhaz (Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta)</td>
<td>TTGAGCAGAGAGCAGGAGGT</td>
<td>GAAGCATTGGGATCAAGAA</td>
</tr>
</tbody>
</table>

Statistics

The results are expressed as means ± standard error of the mean (SEM) and compared to the control group using ANOVA followed by Dunnett’s post hoc analysis. A p-value less than 0.05 was considered significant. Statistical differences were determined on relative value of ATP and ΔCt for mRNA expression.
Results

Liver steatosis

Oil Red O staining revealed an abundance of small-size lipid droplets in PCLS cultured in medium containing additional glucose, fructose and insulin (M4), as well as in PCLS cultured in medium containing additional glucose, fructose, insulin and palmitic acid (M5) at both 24h and 48h (Figure 1). Conversely, lipid-rich droplets were not observed in PCLS cultured in unmodified culture medium (M1), medium containing additional glucose (M2) and medium containing additional glucose and fructose (M3).

Figure 1: Representative Oil Red O-stained sections of PCLS cultured in modified culture media (400X).
To confirm the microscopic evaluation, liver steatosis was quantified by using a fat-ratio calculation (Figure 2). As shown, after 24h and 48h, the fat-ratio of PCLS cultured in M4 increased with 12.7% and 8.5%, respectively, as compared to M1. Likewise, the fat-ratio of PCLS cultured in M5 was 16.3% and 9.1% when compared to M1 after culturing for 24h and 48h, respectively. It should be noted that the fat-ratio of PCLS cultured in M4 was not statistically different from PCLS cultured in M5.

**Figure 2:** Fat-ratio of PCLS cultured in modified culture media. The fat-ratio was determined by measuring the amount of lipid droplets (red area) per nucleus (blue area) using ImageJ software. Data are expressed as means ± SEM (n=3). *p<0.05 compared to unmodified culture medium (M1).

**Viability**

Next, we elucidated whether the induction of steatosis impacted the viability of PCLS (Figure 3). The results showed that the modified media did not influence the viability of PCLS at both 24h and 48h. However, at 48h, the viability of PCLS cultured in M4 was non-significantly reduced compared to control.

**Figure 3:** ATP level/protein of PCLS cultured in modified culture medium at 24h and 48h. Data are expressed as means ± SEM (n=3).
Expression of carbohydrate/lipid metabolism-related genes

Next, we studied change in the expression levels of carbohydrate/lipid metabolism-related genes. As shown in Figure 4, the expression of acetyl-CoA carboxylase 1 (Acc1) was significantly increased 3.1-, 3.7- and 3.4-fold in PCLS cultured for 24h in M3, M4 and M5, respectively. Similarly, the expression of carbohydrate responsive element binding protein (Chrebp) gene was up-regulated 3.4-, 3.2- and 3.0-fold in PCLS cultured in M3, M4 and M5, respectively. Although the gene expression of sterol regulatory element binding protein 1c (Srebp-1c) was increased 2.4-, 3.9- and 3.4-fold in PCLS cultured in M3, M4 and M5, respectively, the differences were not statistically significant. On the other hand, the mRNA level of carnitine palmitoyltransferase 1 (Cpt1) decreased 0.35- and 0.29-fold in PCLS cultured in M4 and M5, respectively. Even though the influence of culture medium modification on the change of carbohydrate/lipid-related gene expression was not statistically different at 48h, the expression profile was similar to the profile at 24h of incubation.

Figure 4: Expression of carbohydrate/lipid metabolism-related genes of PCLS cultured in the modified culture medium at 24h and 48h. Data are expressed as means ± SEM (n=3-5). *p<0.05 compared to M1.
Discussion

Pathophysiological model of NAFLD

To induce liver steatosis, we used supraphysiological concentrations of glucose, fructose, insulin and palmitic acid in the modified culture media to mimic pathophysiological serum concentrations measured in NAFLD patients [11, 22, 25]. Therefore, in addition to the preserved multicellular environment and interplay between various liver cell-types as in the intact liver of PCLS [29], the process in the formation and accumulation of lipid droplets in PCLS could recapitulate the pathophysiological development of the disease. However, it should be noted that it remains to be investigated whether serum levels reflect the portal concentration range of each component.

Insulin acts as the master regulator

Our results demonstrated that insulin is essential for the formation and accumulation of lipid droplets in PCLS [30, 31]. Moreover, a pilot study showed that the steatosis was not observed in PCLS cultured in the medium containing glucose, fructose and palmitic acid, in the absence of insulin (Figure 5). This finding emphasizes again that insulin is the master modulator in the formation and accumulation of lipid droplets in our pathophysiological PCLS model, and probably not palmitic acid. In addition, it is worthwhile to note that palmitic acid did not alter PCLS viability, while clear lipotoxicity was observed at the same concentration in other in vitro studies [19-21]. This discrepancy might be due to the protective effect of insulin which promotes the conversion of free palmitic acid into triglycerides that may accumulate in lipid droplets, thereby decreasing the free fatty acid concentration that might induce lipotoxicity [32].

Disruption of mitochondrial β-oxidation is the main pathway

In our study, the lipid droplets in PCLS can be characterized as microvesicular steatosis which is in line with other in vitro NAFLD studies [33-35]. This differs from the macrovesicular steatosis found in various in vivo models of NAFLD [24, 36, 37]. The main reason why the size of lipid droplets is different in vitro could be the time-frame for induction of steatosis, which in our PCLS, as similar to other in vitro models, are restricted to 48h, and takes many months in in vivo studies. Furthermore, this dissimilarity might also be depended on the main processes for the formation of lipid droplets: mitochondrial β-oxidation and de novo lipogenesis [18, 38]. It was shown in rats-treated with valproate, an anti-epileptic drug, that the development of microvesicular steatosis was due to the mitochondrial β-oxidation impairment [39]. Additionally in clinical practice, microvesicular steatosis is a manifestation in patients who received long-term treatment of various drugs affecting mitochondrial β-oxidation such as tetracycline, amiodarone, aminoptine and pirprofen [40]. On the other hand, macrovesicular steatosis in in vivo models is the result of multiple systemic factors such as eating habits and endocrine effects which chronically and synergistically enhance de novo lipogenesis [23, 24].
The association of both mitochondrial β-oxidation and de novo lipogenesis in the formation and accumulation of lipids in PCLS was supported by the expression profile of gene encoding carbohydrate/lipid-related metabolism. Most importantly, the expression of Cpt1, an enzyme responsible for fatty acid transportation from the cytosol to the mitochondrial matrix where β-oxidation takes places [41], was down-regulated in steatotic PCLS. Furthermore, although the up-regulation of Acc1, Chrebp and Srebp-1c which are key enzymes responsible for the de novo lipogenesis [42-44] was observed in PCLS cultured in medium containing additional glucose and fructose, steatosis was not observed in these liver slices. Taken together, we postulated that the promotion of de novo lipogenesis alone may be insufficient to induce microvesicular steatosis, and therefore the disruption of fatty acid transport and mitochondrial β-oxidation are probably the main pathways responsible for the accumulation of microvesicular steatosis in our PCLS model [45]. Nevertheless, future studies are required to confirm these findings.

Conclusion

Our preliminary study demonstrates that, by mimicking the main risk factors of NAFLD, steatosis can be induced ex vivo in PCLS. The developed model can be an effective tool to study NAFLD and associated conditions, and could possibly be used to test the efficacy of anti-NALFD drugs. Additionally, since PCLS can be prepared from human livers, implementation of a human steatotic PCLS model would be able to overcome some of the limitations of rodent NAFLD/NASH models.
Acknowledgements

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