Hypoxia aggravates non-alcoholic steatohepatitis in mice lacking hepatocellular PTEN

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ABSTRACT

The metabolic disorders that predispose patients to NASH (non-alcoholic steatohepatitis) include insulin resistance and obesity. Repeated hypoxic events, such as occur in obstructive sleep apnoea syndrome, have been designated as a risk factor in the progression of liver disease in such patients, but the mechanism is unclear, in particular the role of hypoxia. Therefore we studied the influence of hypoxia on the development and progression of steatohepatitis in an experimental mouse model. Mice with a hepatocellular-specific deficiency in the Pten (phosphatase and tensin homologue deleted on chromosome 10) gene, a tumour suppressor, were exposed to a 10 % O2 (hypoxic) or 21 % O2 (control) atmosphere for 7 days. Haematocrit, AST (aspartate aminotransferase), glucose, triacylglycerols (triglycerides) and insulin tolerance were measured in blood. Histological lesions were quantified. Expression of genes involved in lipogenesis and mitochondrial β-oxidation, as well as FOXO1 (forkhead box O1), hepcidin and CYP2E1 (cytochrome P450 2E1), were analysed by quantitative PCR. In the animals exposed to hypoxia, the haematocrit increased (60 ± 3% compared with 50 ± 2% in controls; \( P < 0.01 \)) and the ratio of liver weight/body weight increased (5.4 ± 0.2% compared with 4.7 ± 0.3% in the controls; \( P < 0.01 \)). Furthermore, in animals exposed to hypoxia, steatosis was more pronounced (\( P < 0.01 \)), and the NAS [NAFLD (non-alcoholic fatty liver disease) activity score] (8.3 ± 2.4 compared with 2.3 ± 10.7 in controls; \( P < 0.01 \)), serum AST, triacylglycerols and glucose were higher. Insulin sensitivity decreased in mice exposed to hypoxia relative to controls. The expression of the lipogenic genes SREBP-1c (sterol-regulatory-element-binding protein-1c), PPAR-γ (peroxisome-proliferator-activated receptor-γ), ACC1 (acetyl-CoA carboxylase 1) and ACC2 (acyetyl-CoA carboxylase 2) increased significantly in mice exposed to hypoxia, whereas mitochondria β-oxidation genes [PPAR-α (peroxisome-proliferator-activated receptor-α) and CPT-1 (carnitine palmitoyltransferase-1)] decreased significantly. In conclusion, the findings of the present study demonstrate that hypoxia alone aggravates and accelerates the progression of NASH by up-regulating the expression of lipogenic genes, by down-regulating genes involved in lipid metabolism and by decreasing insulin sensitivity.

Key words: β-oxidation, fatty liver, lipogenesis, non-alcoholic steatohepatitis (NASH), peroxisome-proliferator-activated receptor (PPAR), phosphatase and tensin homologue deleted on chromosome 10 (PTEN), sterol-regulatory-element-binding protein-1c (SREBP-1c).

Abbreviations: ACC, acetyl-CoA carboxylase; ALT, alanine aminotransaminase; AST, aspartate aminotransferase; CPT-1, carnitine palmitoyltransferase-1; CYP2E1, cytochrome P450 2E1; FAS, fatty acid synthase; FOXO1, forkhead box O1; H&E, haematoxylin and eosin; HIF-2α, hypoxia-inducible factor-2α; IL-6, interleukin-6; IRS, insulin receptor substrate; MCP-1, monocyte chemotactic protein-1; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; NEFA, non-esterified fatty acid (‘free fatty acid’); OSA, obstructive sleep apnoea; PDK-1, phosphoinositide-dependent kinase-1; PI3K, phosphoinositide 3-kinase; PPAR, peroxisome-proliferator-activated receptor; PTEN, phosphatase and tensin homologue deleted on chromosome 10; SREBP-1c, sterol-regulatory-element-binding protein-1c; TNF-α, tumour necrosis factor-α.

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INTRODUCTION

NASH (non-alcoholic steatohepatitis) is one of the most prevalent liver diseases. It is characterized by steatosis with histological features of hepatocellular inflammation and ballooning and Mallory–Denk bodies. A fraction of patients with NASH progresses to cirrhosis with its attending complications. Numerous factors influence the risk of this development, such as genetic polymorphisms, environmental exposures and co-morbidities. OSA (obstructive sleep apnoea), which is characterized by repetitive episodes of hypoxia during sleep due to short recurrent upper-airway obstruction [1], has been identified as a co-morbid condition. Independent of underlying obesity, OSA is associated with an increased risk of hypertension, Type 2 diabetes, dyslipidemia and atherosclerosis [2]. Several studies link OSA to the pathogenesis of NASH and to the progression of chronic liver injury observed in obese individuals [3,4]. One animal model of diet-induced hepatosteatosis suggested that chronic intermittent hypoxia could indeed cause inflammatory changes and trigger liver injury [5]. However, the influence of hypoxia alone, and not repetitive episodes of hypoxia/re-oxygenation, on the pathogenesis and progression of NASH remains unclear.

Mice lacking the expression of PTEN (phosphatase and tensin homologue deleted on chromosome 10) in their hepatocytes (AlbCrePtenflox/flox mice) develop a phenotype after 10 weeks of age similar to that observed in human NASH [6]. Prominent changes are steatosis, hepatocyte ballooning and a hypersensitivity to insulin. In the present study, we sought to examine the effect of a sustained short-term hypoxia in these animals. AlbCrePtenflox/flox mice at 8 weeks of age, who were predisposed but had not yet developed the NASH phenotype, were exposed to sustained hypoxia for 7 days. The influence of the hypoxic insult on the manifestation and progression of steatohepatitis and insulin sensitivity was examined and the level of expression of several genes involved in lipogenesis was determined. Our results provide evidence that sustained hypoxia aggravates steatohepatitis and support a mechanism whereby hypoxia can drive the progression of fatty liver disease to NASH.

MATERIAL AND METHODS

Animals and experimental procedure
Female 8-week-old AlbCrePtenflox/flox mice [6] were subjected to systemic normobaric hypoxia by substituting O₂ for N₂ using a Digamix 2M 302/a-F pump (Woesthoff Messtechnik) at a constant gas flow rate of 37 litres/min in a closed chamber. To ensure an adaptation period to the hypoxic environment, the $F_{iO_2}$ (fraction of inspired O₂) was gradually decreased from 21 to 10% during the first hour and then kept continuously at 10% O₂ for 7 days. Female 8-week-old AlbCrePtenflox/flox mice exposed to a normal atmosphere (21% O₂) served as controls. Water was provided ad libitum, and food intake was monitored and matched in both groups. Animals were killed immediately after hypoxic exposure, body and liver weights were measured and blood and liver tissue were stored at −80°C for further experiments. This protocol was approved by the Local Animal Use Committee.

Haematocrit level
The haematocrit level was measured in heparinized blood centrifuged in microhaematocrit tubes at 13750 g for 5 min at room temperature (24 °C).

Blood analysis
Plasma levels of triacylglycerols (triglycerides) and the liver transaminases AST (aspartate aminotransferase) and ALT (alanine aminotransaminase) were measured using colorimetric and enzymatic assays (Roche Diagnostics).

Insulin tolerance test
An insulin tolerance test was performed on mice after overnight starvation. Human regular insulin (1 unit/kg of body weight; Actrapid HM; Novo Nordisk) [7] was administered intraperitoneally and blood was collected at 0, 15, 30, 60, 90 and 120 min from tail veins. Glucose was measured using an automated glycaemia reader (Ascensia Contour; Bayer HealthCare).

Histology and immunohistology
Formalin-fixed tissues were embedded in paraffin using standard procedures. Sections (4 μm thick) were cut, stained with H&E (haematoxylin and eosin) for standard microscopy or with Sirius Red to show fibrosis, and were evaluated for steatosis and NASH lesions by a pathologist blinded to treatment conditions as described by Kleiner et al. [8]. The NAS [NAFLD (non-alcoholic fatty liver disease) activity score] was determined as described previously [8]. To visualize lipids, frozen sections (10–20 μm thick) were stained with Oil Red O and counterstained with haematoxylin. The number and the size of lipid droplets were evaluated using the Metamorph software (Molecular Devices). Immunohistochemistry was performed on paraffin-embedded liver sections using an anti-SREBP-1 (sterol-regulatory-element-binding protein-1) antibody (Abcam) and an anti-HIF-2α (hypoxia-inducible factor-2α) antibody (Novus Biologicals) at 1:100 dilutions.

Hepatic triacylglycerol measurement
Total triacylglycerol content in liver tissue was measured by a colorimetric method using the Triglyceride Quantiﬁcation Kit (BioVision).
Table 1  Characteristics and biochemical changes of AlbCrePten^{flox/flox} mice exposed to hypoxia for 1 week

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control (n = 11)</th>
<th>Hypoxia (n = 12)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Female</td>
<td>Female</td>
<td></td>
</tr>
<tr>
<td>Age at start (weeks)</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At start</td>
<td>21.8 ± 0.9</td>
<td>21.7 ± 1.9</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>At end</td>
<td>20.4 ± 0.9</td>
<td>18.6 ± 2.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Liver weight/body weight ratio (%)</td>
<td>4.7 ± 0.3</td>
<td>5.4 ± 0.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Haematocrit level (%)</td>
<td>49.8 ± 1.8</td>
<td>60.1 ± 2.6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AST (IU/l)</td>
<td>61 ± 9</td>
<td>107 ± 22</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>21 ± 5</td>
<td>25 ± 9</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Plasma triacylglycerols (mmol/l)</td>
<td>0.9 ± 0.2</td>
<td>1.8 ± 0.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Plasma glucose (mg/dl)</td>
<td>204 ± 29</td>
<td>305 ± 13</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Real-time quantitative PCR

Total RNA was extracted from liver samples using High Pure RNA Tissue Kit (Roche Applied Sciences) and was reverse-transcribed with MMLV (Mooney-murine-leukaemia virus) reverse transcriptase (Invitrogen). Probes and primers for SREBP-1c (SrebF1), FOXO1 (forkhead box O1; Foxo1), PPAR-γ and PPAR-α [peroxisome-proliferator-activated receptor-γ (Pparγ) and α (Ppara) respectively], CYP2E1 (cytochrome P450 2E1; Cyp2e1), hepcidin (Hamp), ACC1 and ACC2 [acetyl-CoA carboxylase 1 (Acaca) and 2 (Acacb) respectively], CPT-1 (carnitine palmitoyltransferase 1; Cpt1a), FAS (fatty acid synthase; Fasn), MCP-1 (monocyte chemotactic protein-1; Ccl2), TNF-α (tumour necrosis factor α; Tnf) and IL-6 (interleukin 6; Il6) were obtained from TaqMan® Gene Expression assays (Applied Biosystems), and quantitative PCR was performed using an ABI7700 Sequence Detection System and the TaqMan® universal PCR Master Mix, according to standard protocols. The Ct values for each gene were standardized against rRNA (18S) to obtain the ΔCt values. The ΔΔCt values were calculated by subtracting the ΔCt values of mice under normoxia from ΔCt values of animals exposed to hypoxia. Relative fold increases or decreases were calculated using the formula 2^{-ΔΔCt}. All reactions were performed in triplicate.

Statistical analysis

Values are means ± S.D.. Results were compared using the non-parametric Mann–Whitney U test, due to limited sample size. A P value ≤0.05 was considered statistically significant.

RESULTS

Biochemical characteristics of AlbCrePten^{flox/flox} mice exposed to hypoxia

To confirm that the animals were exposed to lower O2 conditions, the haematocrit was measured [10]. This level increased significantly in mice exposed to hypoxia compared with the control group (Table 1). As the food intake decreased initially in mice exposed to hypoxia, but normalized after 3 days, food was restricted in the control group to match consumption of the hypoxic group. Water intake was similar in both groups.

With similar food intake, mice in the control group lost 1.38 g, whereas mice in the hypoxic group lost 3.07 g; however, the liver weight/body weight ratio was increased in the hypoxic group (Table 1). Hypoxia resulted in a significantly increased level of AST, but not ALT, increased triacylglycerols and higher blood glucose compared with the control group (Table 1). After washing, the membranes were incubated for 1 h with a peroxidase-conjugated secondary antibody (Pierce). Immunoblotts were revealed using an enhanced chemiluminescence detection system (PerkinElmer), and exposure was performed with a Fujifilm LAS.100 CCD (charge-coupled-device) camera coupled to a computer. Membranes were re-incubated with an anti-β-actin antibody (Sigma–Aldrich) to control for protein loading. Signals were analysed using the software AIDA 2.1 (Raytest), and protein levels were normalized for β-actin expression.

Western blot analysis

Liver tissue was homogenized in 0.25 mol/l sucrose, and protein concentrations were determined as described by Lowry et al. [9]. Equal amount of proteins (50 μg) were separated by SDS/PAGE and transferred on to nitrocellulose membranes. Membranes were blocked for 1 h with 5% (v/v) non-fat milk and then incubated overnight at 4°C with an anti-CYP2E1 antibody (Abcam), anti-(phospho-S6 ribosomal protein) antibody (Cell Signaling Technology) or anti-(S6 ribosomal protein) antibody (Cell Signaling Technology).
This was accompanied by a smaller reduction in blood glucose level after intraperitoneal insulin injection in mice exposed to hypoxia compared with control animals, as assessed by the insulin tolerance test (Figure 1). These results indicate that hypoxia decreases insulin sensitivity in AlbCrePten^flox/flox mice.

Liver histology

Hepatic histology of animals housed under normal conditions (Figures 2A and 2B, and Table 2) had a moderate steatosis with predominantly microvesicular features. In contrast, AlbCrePten^flox/flox mice exposed to hypoxia (Figures 2C and 2D, and Table 2) had a more pronounced steatosis with a higher frequency of macrovesicles. This was confirmed by Oil Red O staining (Figures 2E and 2F), showing more abundant and larger lipid droplets (Figure 2G), and by the determination of hepatic triacylglycerol content, which was increased (Figure 2H). Fibrosis was absent in all animals as shown by Sirius Red staining (results not shown). More lobular inflammation and small aggregates of macrophages were observed in livers from mice exposed to hypoxia than in control livers. The presence of the occasional balloononed hepatocyte was found only in livers from the hypoxic group. The NAS was also higher in the hypoxic group (Table 2).

Effects of hypoxia exposure on liver gene expression

To investigate the mechanisms leading to the accumulation of triacylglycerols in plasma of AlbCrePten^flox/flox mice exposed to hypoxia, the hepatic expression of several genes involved in lipogenesis was measured by real-time quantitative PCR (Figure 3). The mRNA levels of SREBP-1c, a transcription factor that positively regulates the expression of genes required for lipogenesis [11], were significantly (P < 0.001) increased after hypoxia (Figure 3A). This was confirmed by immunohistochemistry, which showed an increase in the expression of SREBP-1 in the nucleus of hepatocytes exposed to hypoxia (Figures 4A–4D). Among the genes activated by SREBP-1c, ACC catalyses the synthesis of malonyl-CoA, an intermediate in fatty acid synthesis. There are two isoforms of ACC in rodents and humans: ACC1, which is highly expressed in liver and adipose tissue, and ACC2, which is predominantly expressed in heart and skeletal muscle and to a lesser extent in the liver [12]. Both ACC1 and ACC2 genes were significantly (P < 0.001) up-regulated by hypoxia (Figures 3C and 3D), suggesting an increase in malonyl-CoA synthesis. FAS converts malonyl-CoA into palmitoyl-CoA [13], and its gene expression was also significantly (P < 0.005) increased in liver of AlbCrePten^flox/flox mice exposed to hypoxia (Figure 3E). mRNA levels of PPAR-γ, a transcription factor required for normal adipocyte differentiation and that can be transcriptionally activated by SREBP-1c

Table 2: Histological evaluation of steatosis and NASH lesions of AlbCrePten^flox/flox mice exposed to hypoxia for 1 week

<table>
<thead>
<tr>
<th>Evaluation</th>
<th>Control</th>
<th>Hypoxia</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steatosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade</td>
<td>1.8 ± 0.4</td>
<td>2.8 ± 0.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Location</td>
<td>0 ± 0</td>
<td>2.2 ± 1.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Microvesicular steatosis</td>
<td>0.1 ± 0.3</td>
<td>0.5 ± 0.5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Sum</td>
<td>1.9 ± 0.6</td>
<td>5.5 ± 1.9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>Inflammation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lobular inflammation</td>
<td>0.4 ± 0.5</td>
<td>2.0 ± 0.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Microgranuloma</td>
<td>0.1 ± 0.3</td>
<td>0.7 ± 0.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Large lipogranuloma</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>Portal inflammation</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>Ballooning</td>
<td>0.0 ± 0.0</td>
<td>0.8 ± 0.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>NAS score</td>
<td>2.3 ± 0.7</td>
<td>8.3 ± 2.4</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
Hypoxia aggravates NASH

Figure 2  Liver histology in AlbCrePten$^{flox/flox}$ mice exposed to hypoxia for 1 week (C, D and F) in comparison with normoxia controls (A, B and E)

(A and C) Lower magnification ($\times 10$) of H&E-stained livers. Lipid vacuoles were more abundant and larger in the livers of mice exposed to hypoxia. (B and D) Higher ($\times40$) magnification shows the presence of inflammatory cells in the liver of both groups (arrows). Lipid accumulation was confirmed by Oil Red O staining (E and F). (G) Distribution of lipid droplets size evaluated by Metamorph software, with more abundant and larger lipid vacuoles in the livers of AlbCrePten$^{flox/flox}$ mice exposed to hypoxia. (H) Triacylglycerol content measured in the livers of mice exposed to hypoxia and normoxia controls. *$P < 0.005$ compared with the control group ($n = 7$ for the control group and $n = 8$ for the hypoxic group).

[14,15], were also significantly higher ($P < 0.05$) in livers from the hypoxic group compared with control livers (Figure 3B).

The transcription factor FOXO1 has been reported to inhibit the induction of PPAR-$\gamma$ expression and adipocyte differentiation [16]. Hepatic FOXO1 mRNA levels were slightly, but significantly, decreased by hypoxia (Figure 3H).

The effects of hypoxia on mitochondrial $\beta$-oxidation of NEFAs [non-esterified fatty acids (‘free fatty acids’)] were also investigated. PPAR-$\alpha$ regulates mitochondrial, as well as peroxisomal and microsomal, fatty acid oxidation systems in the liver. PPAR-$\alpha$ mRNA levels were significantly reduced in livers of AlbCrePten$^{flox/flox}$ mice exposed to hypoxia (Figure 3F). Hypoxia also reduced mRNA levels of CPT-1 by 40%, the protein responsible for fatty acid transport into mitochondria (Figure 3G). Recently, constitutive activation of HIF-2$\alpha$ was reported to result in impairment of fatty acid $\beta$-oxidation [17]. HIF-2$\alpha$ was increased in the nucleus of hypoxic hepatocytes, as demonstrated by immunohistochemistry (Figures 4E–4H). The mRNA levels of hepcidin, a key regulator of iron homeostasis, were decreased by 80% in mice exposed to hypoxia (Figure 3I). CYP4502E1 is a major source of oxidative stress, and its mRNA and protein expression were increased in the livers of AlbCrePten$^{flox/flox}$ mice exposed to hypoxia (Figures 5A and 5B).
Figure 3  Effect of hypoxia on the expression of liver gene mRNAs measured by real-time quantitative PCR
Genes involved in the synthesis of fatty acids (SREBP-1c, PPAR-γ, ACC1 and ACC2, FAS; A–E) were up-regulated in AlbCrePtenflox/flox mice exposed to hypoxia. Genes involved in mitochondrial β-oxidation (F and G) were down-regulated. FOXO1 gene expression was slightly, but significantly, decreased in the livers of mice exposed to hypoxia (H). Hepcidin mRNA was decreased in the livers of mice exposed to hypoxia (I). *P < 0.05 with control group (n = 10 for the control and hypoxic groups).

As shown in Table 2, lobular inflammation and the presence of aggregates of macrophages were more pronounced in the livers of mice exposed to hypoxia. This increased inflammation state was confirmed by determining the mRNA expression of the pro-inflammatory cytokines MCP-1, TNF-α and IL-6 in liver extracts of AlbCrePtenflox/flox mice (Figures 5C–5E): the mRNA levels of these cytokines were significantly higher in livers from mice exposed to hypoxia.

Mechanism by which hypoxia decreases insulin sensitivity in AlbCrePtenflox/flox mice
To understand the mechanism leading to insulin resistance in AlbCrePtenflox/flox mice exposed to hypoxia, the phosphorylation status of the S6 ribosomal protein was assessed. S6 ribosomal protein belongs to the PI3K (phosphoinositide 3-kinase)/Akt/mTOR (mammalian target of rapamycin) signalling pathway and is phosphorylated by p70 S6 kinase after activation of this pathway. S6 ribosomal protein phosphorylation was increased when AlbCrePtenflox/flox mice were exposed to hypoxia (Figure 6), indicating an activation of p70 S6 kinase. Several studies have shown the existence of a negative feedback loop from p70 S6 kinase to the upstream insulin-responsive IRS (insulin receptor substrate)/PI3K/PDK-1 (phosphoinositide-dependent kinase-1)/Akt pathway [18,19]. Inactivation the IRS proteins by this feedback loop contributes to insulin resistance, explaining the decrease in insulin sensitivity observed in AlbCrePtenflox/flox mice exposed to hypoxia.

DISCUSSION
The main finding of the present study is that chronic hypoxia aggravates steatohepatitis in mice genetically predisposed to NASH. The hypertriglycerolaemia and insulin resistance observed in patients with NASH was reproduced in the AlbCrePtenflox/flox mouse model after exposure to hypoxia. Changes in the hepatic expression of enzymes and transcription factors that regulate key points in the lipid cycle provide a clue as to the mechanism by which hypoxia exerts its detrimental effects. An increased expression of enzymes involved in the synthesis of NEFAs coupled with a decrease in the expression of enzymes leading to mitochondrial β-oxidation appear to impair the overall NEFA metabolism in hypoxic AlbCrePtenflox/flox mice.

Ablation of Pten in mouse hepatocytes results in the spontaneous development of a steatohepatitis in animals older than 10 weeks of age. The livers of 8–10-week-old mice have microvesicular steatosis, but no macrovesicular vacuoles [6]. This pre-NAFLD background afforded us a propitious model in which to investigate effects of hypoxia on the progression of NASH. The liver histology of our control AlbCrePtenflox/flox mice showed little microvesicular steatosis, which can be explained by the fact that female mice are less susceptible to steatotic changes than male mice, as suggested previously [6]. Hypoxia induced a pronounced steatosis with the presence of numerous large lipid droplets (Table 2 and Figure 2) accompanied by lobular inflammation and ballooning, defining features of NASH. It is remarkable that this aggravation of NAFLD occurred despite weight loss. This suggests that hypoxia elicits metabolic effects and is in line with other studies showing that mice with deletion of Vhl (von Hippel–Lindau syndrome), a tumour suppressor promoting the degradation of HIF-1α and HIF-2α, have severe steatosis [20]. Using an intermittent hypoxia model, Polotsky and co-workers
Immunohistochemistry of liver sections of AlbCrePten<sup>flox/flox</sup> mice exposed to normoxia (A, B, E and F) or to hypoxia (C, D, G and H)

(A–D) Immunohistochemical staining of SREBP-1 in livers at lower (A and C) and higher (B and D) magnification, showing increased staining in the nuclei of hepatocytes of mice exposed to hypoxia. 

(E–H) Immunohistochemical staining of HIF-2α in livers at lower (E and G) and higher magnification (F and H), showing increased staining in the nuclei of hepatocytes of mice exposed to hypoxia.

[21] reported that chronic intermittent hypoxia does not cause hepatic steatosis in lean C57BL/6J mice, but does cause steatohepatitis in C57BL/6J mice on a high-fat/high-cholesterol diet [5] and in leptin-deficient obese mice [22], suggesting that intermittent hypoxia aggravates NASH, but is not the sole factor causing NASH. However, the design used by these authors, in contrast with the design of the present study, does not permit a separation of the effects due to hypoxia from those due to cyclic re-oxygenation.

In AlbCrePten<sup>flox/flox</sup> mice, which have been characterized by an increase in insulin sensitivity [6], sustained hypoxia decreased this insulin sensitivity, probably by a mechanism involving the induction of the feedback loop from p70 S6 kinase to the upstream insulin-responsive IRS/PI3K/PDK-1/Akt pathway [18], as indicated by the increase in the phosphorylation of S6 ribosomal protein. Intermittent hypoxia increases insulin resistance not only in leptin-deficient obese mice [23], but also in lean C57BL/6J mice [24]. The severity of OSA is linked to alterations in glucose metabolism [25,26], and insulin resistance has been suggested to promote steatohepatitis in patients with severe OSA [3]. A possible mechanism could be the loss of the repressive effect of insulin on the expression of CYP2E1 [27,28]. CYP2E1 is a major microsomal source of oxidative stress [29] and plays a role in the pathogenesis NASH [30]. CYP2E1 mRNA and proteins levels were increased in the livers of AlbCrePten<sup>flox/flox</sup> mice exposed to hypoxia. Whether increased CYP2E1 is a cause or a consequence of insulin resistance remains unclear. Increased hepatocyte CYP2E1 expression may also result in the down-regulation of
insulin signalling, potentially contributing to the insulin resistance associated with NAFLD [31].

Hepatic overexpression of SREBP-1c in transgenic mice leads to lipogenesis and the development of fatty liver [32]. Insulin can stimulate hepatic SREBP-1c transcription even in the presence of insulin resistance [33]. SREBP-1c activates, among other genes, ACC, which produces malonyl-CoA, an intermediate in fatty acid synthesis, which inhibits CPT-1, the enzyme transferring fatty acyl-CoAs into the mitochondria for β-oxidation. CPT-1 gene expression was decreased by hypoxia in AlbCrePten
\textsuperscript{flx/flx} mice, thus favouring lipogenesis and impairing fatty acid β-oxidation. The combined effect of an increase in SREBP-1c, ACC1, ACC2 and PPAR-γ mRNA levels suggests an activation of fatty acid and triacylglycerol synthesis.

PPAR-α mRNA was reduced by hypoxia, suggesting a decrease in fatty acid catabolism. PPAR-α is highly expressed in the liver and mice lacking PPAR-α develop steatosis [34,35]. Moreover, PPAR-α has anti-inflammatory properties. PPAR-α-null mice have a prolonged inflammatory reaction in response to the inflammatory cytokine leukotriene B4 [36]. PPAR-α suppresses the expression of pro-inflammatory genes, allowing the control and inhibition of inflammation [37]. This suggests that the decrease in PPAR-α mRNA level observed in the liver of AlbCrePten
\textsuperscript{flx/flx} mice exposed to hypoxia may favour inflammation. Moreover, an increase in pro-inflammatory cytokines was observed in the liver of these animals. In contrast with PPAR-α, the expression of PPAR-γ is normally low in the liver, but has been found to be up-regulated in animal models with fatty liver [6,38,39]. Overexpression of hepatic PPAR-γ leads to adipocyte-specific gene expression and lipid accumulation [40]. We observed that hypoxia increased the gene expression of PPAR-γ in the livers of AlbCrePten
\textsuperscript{flx/flx} mice, possibly as a consequence of an increase in SREBP-1c and/or a decrease in FOXO1 gene expression.

Our present results showed a translocation of the transcription factor HIF-2α in the nucleus of hepatocytes exposed to hypoxia. Recently, a link between HIF-2α and hepatic lipid metabolism has been demonstrated [17], where constitutive activation of HIF-2α was associated with impaired fatty acid β-oxidation. Thus hypoxia may affect PPAR-α as well as PPAR-α-target gene expression through the stabilization of HIF-2α and its translocation into the nucleus of hepatocytes, resulting in the decrease of fatty acid catabolism.

Hepcidin is known to be down-regulated by hypoxia [41]. Hepcidin was down-regulated in the livers of 8-week-old animals exposed to low O2 conditions. Hepcidin inhibits iron absorption by enterocytes and iron release from macrophages [42]. By down-regulating hepcidin, hypoxia promotes iron accumulation in the liver, a factor predisposing to oxidative stress, along with the increased expression of CYP2E1.

In our protocol, mice were exposed to continuous hypoxia, which does not mimic the intermittent nature of hypoxia in physiological conditions.
of OSA with its repetitive cycles of hypoxia and re-oxygenation, leading to excessive production of ROS (reactive oxygen species) and oxidative stress in various organs, including the liver [43]. By avoiding these repetitive cycles of hypoxia and re-oxygenation, our model allowed a focussed investigation on the influence of hypoxia alone on the development of steatohepatitis. Although our genetic model of mice lacking hepatocellular PTEN is not a faithful reproduction of the human NASH syndrome, PTEN was found to be down-regulated in hepatocytes by unsaturated fatty acids [44], which implies that hypoxic insults within a cellular environment deficient in PTEN signalling influences the pathogenesis of NASH.

In conclusion, the results of the present study demonstrate that sustained exposure to hypoxia precipitates steatohepatitis in an animal model prone to developing NASH. Hypoxia induced the decrease in insulin sensitivity and changes in the hepatic expression of enzymes promoting the synthesis and inhibiting the degradation of fatty acids participating in this process.

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