RESEARCH

Oxygen therapy alleviates hepatic steatosis by inhibiting hypoxia-inducible factor-2 α

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is difficult to manage due to the lack of effective treatments. Increased oxygen consumption caused by overnutrition, along with reduced oxygen delivery to liver cells induces hepatic steatosis. Here, we investigated the efficacy of oxygen therapy (OT) to alleviate hepatic steatosis. The effect of OT on hepatic steatosis was evaluated in high-fat-diet (HFD)-fed mice and palmitic acid (PA)-treated primary hepatocytes. Liver biopsy tissue samples were used to determine the relationship between the expression of hypoxia-inducible factor-2 α (HIF-2 α) and the progression of NAFLD. The role of HIF-2 α in the OT group was determined based on the overexpression of HIF-2 α *in vitro*. OT safely alleviated hepatic hypoxia and improved hepatic steatosis by inhibiting hepatic *de novo* lipogenesis in HFD-fed mice and PA-treated primary hepatocytes, and this was accompanied by reduced expression of HIF-2 α and hepatic *de novo* lipogenesis. The analysis of liver tissues from individuals with or without NAFLD revealed a positive correlation between hepatic HIF-2 α expression and NAFLD progression. Overexpression of HIF-2 α *in vitro* inhibited the beneficial effect of OT against hepatic lipogenesis and steatosis. OT might be a viable treatment option for NAFLD and functions by alleviating hypoxia and inhibiting the liver HIF-2 α signaling pathway.

Key Words

- ▶ HIF-2 α
- ▶ NAFLD
- ▶ hepatic steatosis
- ▶ oxygen therapy

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Introduction

Non-alcoholic fatty liver disease (NAFLD), a major epidemic chronic liver disease worldwide, is often accompanied by systemic metabolic disorders, such as obesity, type 2 diabetes and cardiovascular diseases (Byrne & Targher 2015, Pais *et al.* 2016). The global average prevalence of NAFLD in adults is 25%, and 15% of these patients might progress from simple steatosis to non-alcoholic steatohepatitis (NASH), increasing the risk of cirrhosis and hepatocellular carcinoma (Febbraio *et al.* 2019). NASH is predicted to become the primary indication for liver transplantation in Western countries

(Cohen *et al.* 2011). Thus, safe and effective treatments for NAFLD are urgently required.

The advocate treatment for NAFLD is weight loss through lifestyle modifications consisting of diet and exercise (Romero-Gomez *et al.* 2017). Weight loss of 3–5% reduces steatosis, and further weight loss (≥ 5 –7%) has been shown to induce the remission of NASH, whereas sustained weight loss of 10% helps to improve hepatic fibrosis (Hannah & Harrison 2016). However, poor compliance often makes it difficult to maintain this long term. Although therapies targeting various pathogenic features

of NAFLD (insulin resistance, hepatic fat accumulation, free radical oxidative stress, inflammation, microbiota and fibrosis) have been widely reported, currently, there is no approved pharmacological treatment to effectively alleviate NAFLD (Rotman & Sanyal 2017).

High oxygen consumption (evidenced by increased mitochondrial respiratory rates due to nutrient overload) and reduced oxygen delivery to liver cells (induced by steatosis) are crucial pathophysiological mechanisms underlying the occurrence and development of NAFLD (Mantena *et al.* 2009, Koliaki *et al.* 2015, Anavi *et al.* 2017). A physiologic oxygen gradient exists in healthy livers, resulting in the metabolic zonation of hepatic lobules and causing hepatocytes in different regions to participate in different types of lipid metabolism to avoid excessive hepatic lipid droplet deposition. However, destruction of the oxygen gradient by hypoxia accelerates hepatic steatosis (Birchmeier 2016, Kietzmann 2017, 2019). Hypoxia-inducible transcription factors (HIFs) are heterodimers that mediate the hypoxia response in the liver, and they are composed of a regulatory HIF- α subunit (HIF-1 α , HIF-2 α and HIF-3 α) and a structural HIF-1 β subunit (HIF-1, HIF-2 and HIF-3) (Koh & Powis 2012).

Experimental studies have indicated that HIF-2 α might be a therapeutic target for NAFLD, as the activation of HIF-2 α (and not HIF-1 α) leads to NAFLD (Qu *et al.* 2011). However, recent studies have suggested that the pharmacological inhibition of HIF-2 α is not a suitable therapy for NAFLD because of the increased risk of elevated postprandial blood glucose, which would aggravate type 2 diabetes (Wei *et al.* 2013, Ramakrishnan *et al.* 2016, Ramakrishnan & Shah 2017). Although hypoxia and increased HIF-2 α expression induce hepatic steatosis, it is unclear whether oxygen therapy (OT) can help to alleviate NAFLD. We conducted this study to determine the efficacy of OT in improving NAFLD *in vivo* and *in vitro* and to uncover the role of HIF-2 α in OT.

Materials and methods

Oxygen therapy

An oxygen chamber containing two small holes and a door was sterilised with 75% ethanol. The OT group of mice or cells was then moved into the oxygen chamber. The oxygen chamber was ventilated through one of the holes with a pipe connected to 100% oxygen for 1 min, and then the door of the oxygen chamber was closed and the other hole was connected to the outside. The oxygen concentration in the chamber was maintained

between 88% and 100% using an oxygen meter based on the reading (Oxygen Meter QD-1100; ESC, Boca Raton, FL, USA). The animal group was subjected to OT at 88% oxygen concentration for 1 h each day at 08:00 h and 20:00 h, and the cell group was subjected to OT at 100% oxygen concentration for 1 h (once).

Animal experimentation

Four-week-old male mice with the C57BL/6J genetic background were obtained from the Model Animal Research Center of Nanjing University, Nanjing, China. The mice were housed in colony cages with 12 h light:12 h darkness cycles, and they had free access to food and water. The study diets were a high-fat diet (HFD: 60% fat, 20% protein and 20% carbohydrates; Research Diets, Inc., New Brunswick, NJ, USA) and normal chow diet (CON: 65% carbohydrate, 24% protein and 11% fat; MediScience Diets Co. Ltd, Yangzhou, China).

After 1 week of adaptation to a normal chow diet, the mice were randomly divided into two groups and fed either the CON ($n=7$) or HFD ($n=14$). After 4 weeks, the mice in the HFD group were randomly divided into the HFD ($n=7$) and OT groups ($n=7$), and the mice in the OT group were provided both HFD and OT (88% oxygen concentration, 1 atm, bid, 1 h each time) for 8 weeks. In the last 2 weeks, the mice were intraperitoneally injected with glucose (2 g/kg body weight) and insulin (1 U/kg body weight) for glucose tolerance testing (GTT) and insulin tolerance testing (ITT), respectively, after fasting for 8 h. Blood samples were collected from the tail veins of mice before and at 30, 60 and 120 min after the injection. All animals were anaesthetised and killed in an isoflurane-filled room 2 days after ITT. The blood oxygen concentration at the outlet of the inferior vena cava near the liver was measured in three anaesthetised mice per group, and the mice in the OT group were administered OT before blood oxygen detection. The blood oxygen concentration was measured using an oxygen probe (OX-NP; a 1.6 $\times$ 40 mm-needle sensor for piercing, Unisense A/S Co., Ltd., Aarhus, Denmark). All animal experiments were performed in accordance with the guidelines and approved by the Institutional Animal Care and Use Committee of Nanjing University.

Primary hepatocyte isolation and culture

Primary hepatocytes were isolated from 4-week-old male mice using a modified two-step collagenase perfusion method. The inferior vena cava of the anaesthetised mice was perfused with 40 mL of Hank's Balanced Salt Solution

(Beyotime, Shanghai, China) for 6 min, and then with 30 mL of Hanks' Balanced Salt Solution with Ca^{2+} and Mg^{2+} (Beyotime) containing collagenase IV (0.2 mg/mL, Cat. no. 17104019; Gibco) for 5 min. The liver was removed, rinsed with PBS and then placed in 10 mL of Hanks' Balanced Salt Solution with Ca^{2+} and Mg^{2+} , shredded, and filtered through a sieve and funnel. The filtrate was added to a 50-mL pre-cooled centrifuge tube and supplemented with 20 mL of Dulbecco's modified Eagle's medium (DMEM) at 4°C and centrifuged at 50 *g* for 2 min, and the pellet was resuspended in DMEM, and this step was repeated three times. Subsequently, 15 mL of pre-chilled medium was added to resuspend the pellet.

The viability of hepatocytes was validated based on trypan blue uptake, and cells with more than 85% viability were used in the subsequent experiments. Hepatocytes were seeded into a six-well plate and cultured in DMEM with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. After 4–6 h of seeding at 37°C under 5% CO_2 in 95% air, humidified atmosphere, the DMEM was changed according to the cell growth conditions. After the adherence of hepatocytes to the wall, they were divided into the following three groups: CON, PA (palmitic acid; Sigma) and OT. PA (0.25 mM) was administered for 24 h, and the OT group was administered oxygen (100% oxygen, 1 atm) in the last hour of PA intervention.

For HIF-2 α overexpression studies, the hepatocytes were divided into the following four groups: PA, OT, lentivirus (LV)-green fluorescent protein (GFP)+OT and LV-HIF-2 α +OT. LV-GFP or LV-HIF-2 α (Genechem, Shanghai, China) was transfected into LV-GFP+OT or LV-HIF-2 α +OT group cells, respectively, at 12 h after seeding. At 48 h post transfection, all the four groups were treated with PA for 24 h, and except for the PA group, the other three groups were subjected to OT in the last hour. The cells were collected for Western blotting and real-time PCR analysis.

H&E and Oil Red O staining

After excising the liver, some tissues were fixed with 4% paraformaldehyde and frozen in liquid nitrogen. Paraformaldehyde-fixed tissues were embedded in paraffin. The prepared paraffin sections were dewaxed in water, washed with distilled water, soaked in hematoxylin stain for 10 min and then rinsed with distilled water to remove excess stain solution. The specimens were rinsed with flowing distilled water for approximately 20 min. After soaking in eosin stain for 10 min, excess eosin was

removed by rinsing with distilled water. The samples were treated with an increasing alcohol gradient (70%, 80% and 90%) for dehydration, followed by sealing with gelatine after routine dehydration.

The frozen tissue was sectioned, fixed with formaldehyde fixing solution for 20 min, washed with distilled water 2–3 times, stained with Oil Red O dye solution for 20 min and immersed in 70% ethanol. After rinsing with distilled water, the sections were counterstained with hematoxylin for approximately 8–10 s. Finally, the sections were sealed with gelatine. The images were captured using the Panoramic 250 slide scanner (3DHistech, Ltd., Budapest, Hungary).

Human subjects

According to the guidance of the Nation Health and Medical Research Council of China, the study with humans was approved by the Ethics Committee of Nanjing Drum Tower Hospital, Nanjing University Medical School. All subjects provided informed consent. The study protocol was performed in accordance with the ethical guidelines of the 1975 Helsinki Declaration. This study has been registered with ClinicalTrials.gov (No. NCT03296605). Frozen liver tissue samples (1.5 × 2.0 × 2.0 cm³) were obtained from patients with NAFLD (*n*=14) who underwent weight-loss metabolic surgery (Roux-en-Y gastric bypass) and from those with hepatic hemangioma without NAFLD (*n*=7). Fourteen patients with NAFLD were divided into the non-alcoholic simple fatty liver (NAFL) group (*n*=7) and the NASH group (*n*=7) based on their NAFLD activity score (NAS), assessed by a pathologist uninformed of the present study. The liver samples were immediately frozen and stored at –80°C until immunohistochemistry and real-time PCR analyses, and patients with hepatitis, cirrhosis, liver cancer or chronic alcohol use were excluded. The clinical and biochemical characteristics of the patients are presented in Table 1.

Biochemical analyses and oxidative stress tests

Cell counting kit-8 (CCK8; Dojindo Laboratories, Kumamoto, Japan) was used for the growth inhibition assay. Total cholesterol, triglycerides (TGs) and high- and low-density lipoprotein cholesterol in mice were measured using ELISA kits according to the manufacturer's instructions (Colorfulgene Biological Technology, Ltd., Wuhan, China). Alanine aminotransferase, aspartate aminotransferase, gamma glutamyltransferase, total cholesterol, TG, high- and low-density lipoprotein

Table 1 Clinical and biochemical characterisation of patients investigated.

Parameters	Non-NAFLD (n = 7)	NAFL (n = 7)	NASH (n = 7)	P-Value
NAS	0	1.1 ± 0.4	5.4 ± 0.5	0
Female/male	4/3	6/1	3/4	0.097
Age (year)	53.9 ± 8.6	33.0 ± 9.9	34.1 ± 11.8	0.003
BMI (kg/m ²)	23.5 ± 2.5	32.0 ± 2.3	37.6 ± 4.1	0.001
SBP (mmHg)	129.9 ± 21.0	129.4 ± 18.4	142.4 ± 30.3	0.826
DBP (mmHg)	82.1 ± 10.2	84.6 ± 14.4	87.7 ± 20.3	0.826
FBG (mmol/L)	4.8 ± 0.7	5.4 ± 1.2	6.8 ± 2.8	0.558
ALT (U/L)	19.3 ± 4.9	18.9 ± 8.4	82.0 ± 36.7	0.003
AST (U/L)	19.2 ± 4.9	16.7 ± 4.5	43.0 ± 22.9	0.003
LDH (U/L)	172.4 ± 25.0	174.6 ± 21.7	191.7 ± 37.3	0.826
γ-GT (U/L)	26.8 ± 10.3	35.4 ± 18.1	51.2 ± 32.2	0.621
TG (mmol/L)	1.4 ± 0.9	3.1 ± 2.2	5.8 ± 4.2	0.558
TC (mmol/L)	4.6 ± 0.8	2.9 ± 1.8	5.8 ± 0.8	0.003
HDL (mmol/L)	1.2 ± 0.3	2.0 ± 1.2	0.8 ± 0.1	0.008
LDL (mmol/L)	2.7 ± 0.6	1.9 ± 0.7	2.5 ± 0.7	0.084
Apolipoprotein A (g/L)	1.2 ± 0.2	1.2 ± 0.3	1.3 ± 0.2	1.000
Apolipoprotein B (g/L)	0.9 ± 0.2	2.4 ± 2.4	1.0 ± 0.3	0.263

Data are presented as mean ± s.d.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; DBP, diastolic blood pressure; FBG, fasting blood glucose; GGT, gamma glutamyltransferase; HDL, high-density lipoprotein (HDL); LDH, lactate dehydrogenase; LDL, low-density lipoprotein (HDL); NAFL, non-alcoholic simple fatty liver; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride.

cholesterol, apolipoprotein A and apolipoprotein B in patients were measured in the Department of Laboratory Medicine, Nanjing Drum Tower Hospital, Nanjing University Medical School, Nanjing, China. The liver and cell TG levels were determined using reagent kits (Beijing Puli Lai Company, Beijing, China). Superoxide dismutase, glutathione peroxidase and malondialdehyde were determined by ELISAs (Beyotime).

Immunohistochemistry

Human liver samples were washed with PBS, fixed in paraformaldehyde for 24 h, embedded in paraffin and then sliced into 4-μm sections. The sections were immunostained with the anti-HIF-1α antibody (diluted 1:1000 v/v; Abcam) and anti-HIF-2α antibody (diluted 1:100 v/v; Abcam). Brown staining was considered as a positive result. Images were captured using the Panoramic 250 slide scanner (3DHitech Ltd.).

Western blotting and real-time PCR

Total protein was extracted using RIPA lysis buffer. For insulin-signaling experiments, the primary hepatocytes were pre-treated with 100 nmol/L insulin for 15 min (Sigma-Aldrich) before protein extraction. The protein samples were separated by sodium dodecyl sulphate-PAGE and transferred onto a polyvinylidene difluoride

membrane (Millipore) and incubated with primary antibodies against HIF-1α (diluted 1:1000 v/v; Abcam), HIF-2α (diluted 1:100 v/v; Abcam), AKT (diluted 1:1000 v/v; Cell Signaling Technology) and phospho-AKT (Ser473) (pAKT, diluted 1:1000 v/v; Cell Signaling Technology). All samples were evaluated by re-probing with anti-GAPDH (diluted 1:4000 v/v; Cell Signaling Technology) to verify equal protein loading.

Total RNA was isolated using TRIzol reagent (Invitrogen) and reverse-transcribed using the cDNA RT Kit (Takara BIO). Real-time PCR was performed on a Light Cycler 480 (Roche) with the following cycling conditions: 95°C for 10 min, 45 cycles of 95°C for 15 s and 60°C for 1 min. Relative gene expression was normalised to levels of *β-actin* as the housekeeping gene and calculated as $2^{-\Delta\Delta C_t}$. The primers used are listed in Table 2.

Inhibition of translation and proteasome inhibition

To detect protein synthesis levels, primary hepatocytes were respectively plated in two six-well plates and divided into a control group (OT 0 h) and an oxygen therapy group (OT 1 h), both of which were treated with PA for 24 h, and the OT group was administered oxygen therapy for 1 h. The translation inhibitor cycloheximide (0.1 mg/mL, Sigma) was added to each cell group for 0, 15, 30, 45 or 60 min, respectively, and the proteins were collected for Western blotting. To detect proteasomal

Table 2 Oligonucleotide sequences of primers used for real-time PCR.

Gene	Forward primer	Reverse primer
<i>β-actin</i> (human)	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT
<i>HIF-1α</i> (human)	GAACGTCGAAAAGAAAAGTCTCG	CCTTATCAAGATGCGAACTCACA
<i>HIF-2α</i> (human)	CGGAGGTGTTCTATGAGCTGG	AGCTTGTGTGTTTCGACAGGAA
<i>β-actin</i> (mouse)	CCACAGCTGAGAGGGGAAATC	AAGGAAGGCTGGAAAAGAGC
<i>Hif-1α</i> (mouse)	TCTCGGCGAAGCAAAGAGTC	AGCCATCTAGGGCTTTTCAGATAA
<i>Hif-2α</i> (mouse)	GAGGAAGGAGAAATCCCGTGA	TATGTGTTCCGAAGGAAGCTGA
<i>Srebp1c</i> (mouse)	TGACCCGGCTATTCCGTGA	CTGGGCTGAGCAATACAGTTC
<i>ChREBP</i> (mouse)	AGATGGAGAACCACGATATCA	ACTGAGCGTGCTGACAAGTC
<i>Acc</i> (mouse)	CTCCCATTATCAATTGGGTCTG	TCGACCTTGTTTTACTAGGTGC
<i>Fasn</i> (mouse)	GGAGGTGGTGATAGCCGGTAT	TGGGTAATCCATAGAGCCAG
<i>Scd1</i> (mouse)	TTCTTGCGATACACTCTGGTGC	CGGGATTGAATGTTCTTGTCGT
<i>Dgat1</i> (mouse)	GTGCCATCGTCTGCAAGATTCT	GCATCACCACACCAATTCAG
<i>Dgat2</i> (mouse)	GCGCTACTTCCGAGACTCTT	GGGCCTTATGCCAGGAAGT
<i>Cidea</i> (mouse)	TGACATTCATGGGATTGCAGAC	TGACATTCATGGGATTGCAGAC
<i>Elovl6</i> (mouse)	GAAAAGCAGTTCAACGAGAAGC	AGATGCCGACCACCAAAGATA
<i>Plin2</i> (mouse)	CTTGTGTCCTCCGCTTATGTC	GCAGAGGTCACGGTCTTCAC

Acc, acetyl coenzyme A carboxylase; ChREBP, carbohydrate response element binding protein; Cidea, cell death-inducing DFFA-like effector A; Dgat1, diacylglycerol O-acyltransferase 1; Dgat2, diacylglycerol O-acyltransferase 2; Elovl6, ELOVL fatty acid elongase 6; Fasn, fatty acid synthase; *HIF-1α*, hypoxia-inducible factor-1α; *HIF-2α*, hypoxia-inducible factor-2α; *Plin2*, perilipin 2; *Scd1*, stearoyl-CoA desaturase; *Srebp1c*, sterol response element-binding protein 1c.

degradation, primary hepatocytes were divided into the following four groups: CON, OT, MG132 and OT+MG132, which were all treated with PA for 24 h. The OT and OT+MG132 groups were supplied with oxygen for 1 h, and the MG132 and OT+MG132 groups were treated with the proteasomal inhibitor MG132 (0.01 mmol/L, Sigma) for 1h. The proteins were collected for Western blotting.

Data analysis and statistical calculations

Statistical analysis was performed with GraphPad (version 7.0, GraphPad, Inc.) and SPSS (version 22.0, SPSS, Inc.). The results are expressed as the mean ± s.d. and as proportions (qualitative data). The statistical differences were analysed by one-way ANOVA with Tukey's post hoc test for multiple comparisons or chi-square tests for categorical variables. $P < 0.05$ was considered statistically significant.

Results

OT improves hepatic steatosis

To verify whether OT can improve oxygen flow in the liver, we measured the blood oxygen concentration at the outlet of the inferior vena cava near the liver using an oxygen probe. The results revealed that OT significantly increased the blood oxygen concentration (Fig. 1A) and decreased the body weight and liver/body weight ratios in

HFD-administered mice (Fig. 1B, C, D and E). Hepatic steatosis due to the HFD was improved, as evidenced by decreased hepatic TG levels and reduced sizes and numbers of lipid droplets based on H&E- and Oil Red O-stained hepatic tissue sections (Fig. 1F and G), which was accompanied by the alleviation of dyslipidaemia (Fig. 1H). Furthermore, OT significantly improved hepatocellular steatosis and reduced intracellular TG levels in PA-treated primary hepatocytes ($P < 0.05$; Fig. 1I and J).

We next investigated the effect of OT on glucose metabolism and insulin resistance by performing GTTs and ITTs. The results revealed that OT lowered HFD-induced hyperglycaemia and increased insulin sensitivity (Fig. 2A and B). OT also stimulated the insulin-signaling pathway, as evidenced by the increase in hepatocyte insulin-stimulated Akt phosphorylation levels with PA (Fig. 2H). The levels of oxidative stress indicators, namely malondialdehyde and glutathione peroxidase, in the livers of HFD mice, as well as superoxide dismutase in PA-treated primary hepatocytes, were also decreased by OT (Fig. 2C, D and E). Furthermore, OT increased the proliferation of primary hepatocytes, as determined using CCK8 ($P < 0.05$; Fig. 2F) and reduced the obvious swelling of mitochondria and mitochondrial cleavage in PA-treated primary hepatocytes (Fig. 2G).

HIF-2α but not HIF-1α is related to NAFLD progression

To investigate the association between HIF-1α/HIF-2α and NAFLD, we evaluated the expression of HIF-1α and HIF-2α in the liver tissues of non-NAFLD group individuals

Figure 1

Oxygen therapy (OT) improves hepatic steatosis in high fat diet (HFD)-fed mice and in palmitic acid (PA)-pre-treated primary mouse hepatocytes. Oxygen content in the inferior vena cava from the CON (control), HFD and OT groups ($n = 3$ subjects/group) (A). Liver morphology (B), gross morphology (C), body weight (D), liver-to-body weight ratio (E) and hepatic triglyceride (TG) levels (F). Representative H&E- and Oil Red O-stained liver sections (magnification $\times 20$) (G). Serum total cholesterol (TC), TG, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) levels (H). Oil Red O staining of primary hepatocytes from the CON, PA and OT groups ($n = 3$ subjects/group) (I). Hepatocyte TG levels (J). Values are the mean $\pm$ s.d. ($n = 7$ animals/group). $*P < 0.05$, $**P < 0.01$. Data are represented as the mean $\pm$ s.d. A full color version of this figure is available at <https://doi.org/10.1530/JOE-19-0555>.

(patients with hepatic hemangioma, $n = 7$) and individuals with NAFLD ($n = 14$) by immunohistochemistry (Fig. 3A and C) and real-time PCR (Fig. 3B). The results suggested that compared to those in patients without NAFLD, patients with NAFLD had significantly higher HIF-2 α levels in the cytoplasm and nuclei of hepatocytes, and the hepatic gene and protein expression of HIF-2 α was associated with the progression of NAFLD. Increased hepatic gene and protein expression of HIF-1 α was

confirmed in patients with NAFLD, but it did not correlate with the progression of disease.

OT reduces hepatic lipogenesis and HIF-2 α mediates the alleviation of hepatic steatosis induced by OT

To further explore how OT might improve hepatic steatosis, we tested lipid synthesis-related genes before and after OT *in vivo* and *in vitro*. We found that OT

Figure 2

Oxygen therapy (OT) improves glucose metabolism and insulin sensitivity in high fat diet (HFD)-fed mice, alleviates hepatic oxidative stress and improves hepatocellular viability and mitochondrial structure. Glucose tolerance testing (GTT; A) and insulin tolerance testing (ITT; B). * $P < 0.05$, ** $P < 0.01$ vs CON (control) group, # $P < 0.05$, ## $P < 0.01$ vs HFD group. Hepatic oxidative stress indicators MDA and GSH-Px in the CON, HFD and OT groups and SOD levels in primary hepatocytes (C, D and E). * $P < 0.05$. Primary hepatocyte proliferation, as measured by CCK8 assays (F). * $P < 0.05$, vs CON group, # $P < 0.05$ vs PA (palmitic acid) group. Mitochondrial morphology analysis (arrow) based on transmission electron microscopy images of primary hepatocytes and quantification of mitochondrial volume density and number (G). Scale bar = 5.0 μm . * $P < 0.05$. Western blotting images of AKT and pAKT (Ser473) expression before and after insulin stimulation among CON, PA and OT groups (H). * $P < 0.05$, ** $P < 0.01$. Data are represented as the mean $\pm$ s.d. A full color version of this figure is available at <https://doi.org/10.1530/JOE-19-0555>.

Figure 3

Hepatic HIF-2α is overexpressed with the development and progression of non-alcoholic fatty liver disease (NAFLD) in humans. Representative immunohistochemical staining for the expression of HIF-1α and HIF-2α in human hepatic tissue from non-NAFLD, NAFL and non-alcoholic steatohepatitis (NASH) patients ($n = 7$ subjects/group, magnification $\times 80$) (A). mRNA expression levels and immunohistochemistry analysis of HIF-1α and HIF-2α in human hepatic tissue (B and C). Not significant (n.s.) $P > 0.05$, * $P < 0.05$, ** $P < 0.01$. Data are represented as the mean $\pm$ s.d. A full color version of this figure is available at <https://doi.org/10.1530/JOE-19-0555>.

down-regulated genes that are associated with hepatic *de novo* lipogenesis in HFD mice, such as sterol response element-binding protein 1c (*Srebp1c*), carbohydrate response element binding protein (*ChREBP*), acetyl coenzyme A carboxylase (*Acc*), fatty acid synthase (*Fasn*), stearoyl-CoA desaturase (*Scd1*), diacylglycerol O-acyltransferase (*Dgat1*, *Dgat2*), cell death-inducing DFFA-like effector A (*Cidea*), ELOVL fatty acid elongase 6 (*Elovl6*) and perilipin 2 (*Plin2*), and similar results were obtained *in vitro* (Fig. 4A). We also found that OT decreased the hepatic gene and protein expression of HIF-2 α due to HFD and PA *in vivo* and *in vitro* (Fig. 4B and C). To verify whether the reduction in HIF-2 α is a key molecular mechanism through which OT alleviates hepatic steatosis, *HIF-2 α* overexpression in primary hepatocytes was achieved through lentivirus transduction. The results showed that the improvement in hepatic steatosis mediated by

OT was diminished by the overexpression of *HIF-2 α* , as shown by Oil Red O staining (Fig. 4D) and TG levels (Fig. 4E). Furthermore, the overexpression of *HIF-2 α* inhibited the suppressive effect of OT on genes associated with hepatic *de novo* lipogenesis in primary hepatocytes treated with PA (Fig. 4F). Finally, OT was confirmed to decrease HIF-2 α levels by reducing the synthesis of HIF-2 α and promoting the ubiquitination/degradation of HIF-2 α in primary hepatocytes (Fig. 4G and H) and thus inhibited hepatic lipogenesis.

Discussion

HIF-2 α activation, promoted by hepatic hypoxia, plays a pivotal role in hepatic steatosis. In the present study, we verified that OT effectively and safely improves

Figure 4

Oxygen therapy (OT) down-regulates gene and protein levels related to hepatic lipogenesis and HIF-2 α *in vivo* and *in vitro*, and HIF-2 α overexpression inhibits the effect of OT on hepatic steatosis and lipogenesis. Expression of hepatic lipogenesis genes in mice and primary hepatocytes (A). * $P < 0.05$ vs CON (control) group, # $P < 0.05$ vs high-fat diet (HFD) or palmitic acid (PA) group. Hepatic gene (B) and protein (C) expression levels of HIF-1 α and HIF-2 α in mice and primary hepatocytes. * $P < 0.05$, ** $P < 0.01$. Oil Red O staining (D) and hepatocyte triglyceride levels (E) in the PA, OT, LV-GFP + OT and LV-HIF-2 α + OT groups of primary hepatocytes ($n = 3$ subjects/group). * $P < 0.05$, ** $P < 0.01$. Hepatic expression levels of hepatic lipogenesis genes among PA, OT, LV-GFP + OT or LV-HIF-2 α + OT groups ($n = 4$ subjects/group) (F). * $P < 0.05$ vs PA group, # $P < 0.05$ vs OT group. The degradation rate constants of HIF-2 α proteins at 0, 15, 30, 45 and 60 min after cycloheximide (CHX) treatment with or without OT in primary hepatocytes (G) * $P < 0.05$ vs 0 min without OT, # $P < 0.05$ vs 0 min with OT. The protein levels of HIF-2 α with MG132, OT or MG132 and OT in primary hepatocytes (H). * $P < 0.05$. Data are represented as the mean $\pm$ s.d. A full color version of this figure is available at <https://doi.org/10.1530/JOE-19-0555>.

HFD-induced NAFLD and glucolipid metabolism disorders in a mouse model, as well as hepatic steatosis induced by PA. Decreased hepatic HIF-2 α expression induced by OT might thus be beneficial.

Hepatic hypoxia facilitates and aggravates NAFLD. In a previous study, *ob/ob* mice developed NAFLD at an earlier age when raised in an environment of chronic intermittent hypoxic environment (Li *et al.* 2005). Furthermore, exposure to a hypoxic atmosphere was found to accelerate the progression of NASH in hepatocellular *Pten* knockout mice and HFD-fed C57BL/6 mice (Savransky *et al.* 2007, Piguet *et al.* 2009). Hepatic cells cultured under a hypoxic environment also exhibit steatosis (Savransky *et al.* 2007, Piguet *et al.* 2009). We found that hepatic hypoxia and hepatic steatosis in NAFLD mice caused by HFD were improved by OT, and accordingly, improvements in the hepatocyte insulin-signaling pathway induced by OT play a role in hepatic steatosis.

Hypoxia up-regulates the hepatic lipogenesis-related genes *Srebp1c* and *Acc* in obese mice with hepatic steatosis and activates pro-inflammatory cytokines interleukin (*IL*)1 and *IL*6 in NASH mice (Li *et al.* 2005, Piguet *et al.* 2009). We found that OT decreased the expression of hepatic *de novo* lipogenesis genes in NAFLD mice and PA-pre-treated primary hepatocytes, indicating that it can suppress hepatic steatosis by reducing hepatic lipid synthesis, thereby inhibiting the progression of NAFLD.

HIF-1 α and HIF-2 α are both key messenger proteins that mediate hepatocyte metabolism upon hypoxia exposure. HIF-1 α is mainly involved in the short-term severe hypoxia response and HIF-2 α is a major anoxic response factor involved in long-term mild hypoxia,

such as NAFLD (Koh & Powis 2012). Our findings are similar to those of other studies, that is, hepatic HIF-2 α and not HIF-1 α is specifically increased and overexpressed during the development and progression of NAFLD in humans and mice and that HIF-2 α expression is increased in lipid droplet-containing hepatocytes induced by PA (Xie *et al.* 2017, Morello *et al.* 2018). We also found that OT suppressed hepatic HIF-2 α expression and improved hepatic steatosis *in vivo* and *in vitro*. Furthermore, we confirmed that OT decreases the synthesis of HIF-2 α and promotes the ubiquitination/degradation of HIF-2 α *in vitro*. Conversely, up-regulating HIF-2 α attenuated the OT-mediated effects of alleviating lipid deposition and suppressing lipogenesis *in vitro*. Thus, hypoxia following increased lipid droplet accumulation in hepatocytes might induce the overexpression of HIF-2 α , further promoting lipid deposition and aggravating hepatic hypoxia. This cycle could perpetuate the development and progression of NAFLD and can be disrupted by OT.

Hyperbaric (2.5 atm) OT slightly improves hepatic steatosis in NASH mice but aggravates inflammation and promotes aberrant TNF α expression. However, liver oxidative stress was found to be relieved by this treatment in ApoE-knockout mice in other studies (Kudchodkar *et al.* 2007, Tsuneyama *et al.* 2011). In the present study, we confirmed that 1 atm of OT alleviates hepatic oxidative stress and improves hepatocellular viability and mitochondrial structure. This indicated that although OT relieves hepatic steatosis, a suitable concentration and pressure of OT should be determined to avoid aggravating hepatic inflammation. In addition, although we found that OT inhibited hepatic *de novo* lipogenesis by reducing HIF-2 α expression and improved glycometabolism and the hepatic insulin signaling pathway, the detailed mechanism underlying this effect requires further analysis. Another limitation of the present study was the lack of a local liver-targeting OT experiment; thus, we could not exclude the fact that general OT might also affect NAFLD by improving hypoxia in other organs. Finally, we did not detect mitochondrial respiratory rates in the present study; however, in other studies, up-regulated hepatic mitochondrial respiration was confirmed in patients with NAFLD and in HFD-diet-induced NAFLD mice, and the development of mitochondrial dysfunction with the progression of NAFLD was clarified in these studies. The results indicated higher hepatocyte oxygen consumption in the early phase of NAFLD and a loss of hepatic mitochondrial functional adaptation with the progression of NAFLD (Anavi *et al.* 2017). The obvious swelling of the mitochondria and mitochondrial cleavage

Figure 5

Schematic representation of the mechanisms underlying the effects of oxygen therapy on non-alcoholic fatty liver disease (NAFLD). Oxygen therapy alleviates hepatic lipogenesis and steatosis by inhibiting HIF-2 α . A full color version of this figure is available at <https://doi.org/10.1530/JOE-19-0555>.

in PA-treated primary hepatocytes in the present study support the previous results.

In conclusion, we found that OT safely and effectively improves hepatic hypoxia and steatosis, improves glucolipid disorders related to NAFLD in HFD mice, and decreases lipid droplets in hepatocytes pre-treated with PA. The inhibition of hepatic HIF-2 α expression mediated by OT might thus play a vital role in the treatment of NAFLD (Fig. 5). We determined the effect of OT on NAFLD, indicating that OT can be used as a new and effective therapy for NAFLD.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

L Y contributed to the study design, data collection and analysis and drafting of the manuscript. H R W contributed to the technical support of oxygen therapy. X X H and H H L contributed to the technical support of animal experiments. D L Z contributed to the data interpretation. J H W contributed to the study design and the technical support of oxygen therapy. Y B and W H F contributed to the study design and manuscript revision. The final version of the manuscript was read and approved by all authors.

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