Original Article

Hepatic *Cyp1a2* Expression Reduction during Inflammation Elicited in a Rat Model of Intermittent Hypoxia

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Abstract

Background: Intermittent hypoxia (IH) is a key element of obstructive sleep apnea (OSA) that can lead to disorders in the liver. In this study, IH was established in a rat model to examine its effects on the expression of hepatic cytochrome P450 (CYP) and CYP regulators, including nuclear receptors.

Methods: Hematoxylin and eosin staining was conducted to analyze the general pathology of the liver of rats exposed to IH. The messenger RNA (mRNA) expression levels of inflammatory cytokines, CYPs, nuclear factor-κB (NF-κB), and nuclear factors in the liver were measured by quantitative reverse transcription polymerase chain reaction.

Results: We found inflammatory infiltrates in the liver of rats exposed to IH. The mRNA expression level of interleukin-1beta was increased in the liver of the IH-exposed rats (0.005 ± 0.001 vs. 0.038 ± 0.008, $P = 0.042$), whereas the mRNA expression level of *Cyp1a2* was downregulated (0.022 ± 0.002 vs. 0.0050 ± 0.0002, $P = 0.029$). The hepatic level of transcription factor NF-κB was also reduced in the IH group relative to that in the control group, but the difference was not statistically significant and was parallel to the expression of the pregnane X receptor and constitutive androstane receptor. However, the decreased expression of the glucocorticoid receptor upon IH treatment was statistically significant (0.056 ± 0.012 vs. 0.032 ± 0.005, $P = 0.035$).

Conclusions: These results indicate a decrease in expression of hepatic CYPs and their regulator GR in rats exposed to IH. Therefore, this should be noted for patients on medication, especially those on drugs metabolized via the hepatic system, and close attention should be paid to the liver function of patients with OSA-associated IH.

Key words: Cytochrome P450; Glucocorticoid Receptor; Inflammation; Intermittent Hypoxia; Nuclear Factor-κB

Introduction

Obstructive sleep apnea (OSA) is a disorder characterized by the repeated collapse of the upper airway in sleep, which leads to intermittent hypoxia (IH). Epidemiologic studies have proved this common condition to have a prevalence rate of 5–15%.[1] OSA-associated IH induces repetitive cycles of hypoxia and reoxygenation, leading to the generation of reactive oxygen species (ROS) and systemic oxidative stress, which are associated with all manifestations of the metabolic syndrome, including hypertension, insulin resistance, glucose intolerance, and dyslipidemia.[2] Recent evidence in humans and animals have indicated that IH also leads to liver injury.[3,4]

As one of the largest metabolic organs in the body, the liver is the center of nutrient synthesis, toxin resolution, and drug metabolism. The hepatic cytochrome P450 (CYP) plays a vital role in the bioactivation or inactivation of a wide range of xenobiotics.[5] Families 1–3 constitute almost half

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of the total CYPs in mammals and generally catalyze a diverse spectrum of reactions, including the metabolism of endogenous compounds, such as cholesterol, and exogenous compounds, such as drugs.[6] IH has been strongly suggested as an independent risk factor for the severity of liver fibrosis and fibroinflammation,[7] which can cause nonalcoholic fatty liver disease.[8] Oxidative stress and inflammation might be key factors contributing to the development of liver injury under hypoxemic conditions. The inflammatory transcription factor nuclear factor-κB (NF-κB) is activated in patients suffering from OSA, where its activity is correlated with the severity of the sleep condition.[9] Moreover, a hypoxic environment can lead to the expression of more proinflammatory factors, such as interleukin-1beta (IL-1β), IL-6, and tumor necrosis factor-alpha (TNF-α). However, the effects of these inflammatory and proinflammatory factors on IH-related liver injury have not been well examined. Besides the pathologic changes, IH may also have an effect on hepatic CYP activity, further influencing the metabolism of many drugs. A significant correlation has been found between hepatic CYP2E1 activity and nocturnal hypoxia.[10] We had previously reported that rats exposed to IH and cigarette smoke exhibited an increase of hepatic inflammatory cytokines, a reduction of CYPs, and a decrease of the nuclear receptors pregnane X receptor (PXR), constitutive androstane receptor (CAR), and glucocorticoid receptor (GR).[11] Therefore, we hypothesized that IH-associated inflammation will independently lead to liver injury, which may further be exacerbated by another hepatic insult. To test this hypothesis, we exposed male Wistar rats to IH and examined the effects on inflammatory cytokine and CYP expression in the liver, and we also explored the possible mechanism behind these effects.

Methods

Ethical approval

Rats were used under the strict protocol approved by the Animal Care Committee of Haihe Clinical College of Tianjin Medical University (Permit Number: 2010-0002).

Materials

Six-week-old male Wistar rats weighing 180 ± 20 g were purchased from the Laboratory Animals Center of the Institute of Radiation Medicine, Chinese Academy of Medical Sciences & Peking Union Medical College (Tianjin, China). The gas oxygen concentration monitor was purchased from Hamilton Medical AG (Bonaduz, Switzerland). The flow and velocity meter was purchased from Instrument and Meter Plant (Yuyao, China). The blood-gas analyzer (model AVL 995) was purchased from Roche (Basel, Switzerland). Air and/or nitrogen gas circulation was achieved by housing the animals in modified glass boxes, and a low-oxygen tank was reformed by using a large sealed box. The G-Storm Gradient polymerase chain reaction (PCR) thermal cycler was purchased from Bio-Rad Laboratories (Hercules, CA, USA), and the LightCycler Real-Time PCR system was purchased from Roche. The RNA concentration meter was purchased from APG BIO Ltd., (Shanghai, China). TRIZOL Reagent was purchased from Invitrogen (Carlsbad, CA, USA). The TIANScript RT kit was purchased from TIANGEN Biotech Ltd., (Beijing, China). SYBR Green PCR core reagents were purchased from Bio-Rad Laboratories. Established programs were used to control the cycle index and cycle time of IH and intermittent normoxia (IN).

Groups and modeling

Thirty male Wistar rats were separated into two groups of 15 animals each according to oxygen exposure conditions. Both groups were bred normally for 8 weeks. On the 9th week, for 8 h during sleep time (9:00–17:00) every day, the rats in the control group were exposed to cycles of air (IN condition), whereas the rats in the IH group were exposed to cycles of nitrogen followed by air. Each cycle lasted for 120 s (30 cycles per hour), where that of IH involved 30 s of nitrogen followed by 90 s of air. The IH treatment was continued for 6 weeks.

Liver tissue sampling

At the end of the IH treatment, all rats were anesthetized and sacrificed. The abdominal cavity was opened, and the liver tissues were excised, rinsed in ice-cold PBS (pH 7.4), and then either stored at −80°C for gene expression analysis or fixed in 10% neutral-buffered formalin for histologic analysis.

Hematoxylin and eosin staining

Lung and liver samples were fixed in 10% neutral-buffered formalin. After trimming, the tissues were embedded in paraffin using a tissue processor, and 3–4-μm-thick sections were cut. The sections were then stained with hematoxylin and eosin (H and E) solution for visualization by microscopy.

Preparation of RNA from tissue samples

RNA was extracted from the liver tissues using TRIZOL Reagent. The extract yield and quality were determined by measuring the absorbance at 260 and 280 nm with the MaestroNano Micro-Volume Spectrophotometer (Maestrogen, Inc., Las Vegas, NV, USA). The 260:280 absorbance ratio was between 1.8 and 2.0. The RNA was subsequently reverse transcribed into complementary DNA (cDNA).

Quantitative reverse transcription polymerase chain reaction

Messenger RNA (mRNA; 3 μg) was reverse transcribed with oligo (dT) primers for 1 h at 50°C, using the TIANScript RT kit according to the manufacturer's instructions. The cDNAs served as templates for the quantitative reverse transcription polymerase chain reaction, which was performed using SYBR Green PCR core reagents. Specific gene primers were designed using Primer-Quest SM software available at http://www.idtdna.com/Scitools/Applications/PrimerQuest/ (Integrated DNA Technologies, Inc., Coralville, IA, USA) and then produced commercially (BGI Tech, Shenzhen, China) [Table 1]. DNA amplifications were performed on a CFX96 Real-Time system (Bio-Rad Laboratories) at the

Table 1: Primer sequences used for gene amplification

Gene	Probe	Sequence
IL-1β	Forward primer	5'-TCCCTGAACTCAACTGTGAAATA-3'
	Reverse primer	5'-GGCTTGGAAGCAATCCTTAATC-3'
IL-6	Forward primer	5'-GAAGTTAGAGTCACAGAAGGAGTG-3'
	Reverse primer	5'-GTTTGCCGAGTAGACCTCATAG-3'
TNF-α	Forward primer	5'-ACCTTATCTACTCCCAGGTTCT-3'
	Reverse primer	5'-GGCTGACTTTCTCCTGGTATG-3'
PXR	Forward primer	5'-GAAGATCATGGCTGTCCTCAC-3'
	Reverse primer	5'-CGTCCGTGCTGCTGAATAA-3'
CAR	Forward primer	5'-GAGACCATGACCAGTGAAGAAG-3'
	Reverse primer	5'-AGTCAGGGCATGGAAATGATAG-3'
GR	Forward primer	5'-CAGCAGTGAAATGGGCAAAG-3'
	Reverse primer	5'-GGGCAAATGCCATGAGAAAC-3'
Cyp1a2	Forward primer	5'-GACAAGACCCTGAGTGAGAAG-3'
	Reverse primer	5'-GAGGATGGCTAAGAAGAGGAAG-3'
Cyp2c9	Forward primer	5'-CCCAAGGGCACAACCATATTA-3'
	Reverse primer	5'-CTTTCTGGATGAAGGTGGCA-3'
Cyp2c19	Forward primer	5'-CCCAAGGGCACAACCATATTA-3'
	Reverse primer	5'-TTTGACCCTCGTCACTTTCTG-3'
Cyp2d4	Forward primer	5'-CCTTTCAGCCCTAACACTCTAC-3'
	Reverse primer	5'-ATGAAGCGTGGGTCATTGT-3'
Cyp3a2	Forward primer	5'-GGAAACCCGTCTGGATTCTAAG-3'
	Reverse primer	5'-GAAGTGTCTCATAAAGCCCTGT-3'
Gapdh	Forward primer	5'-ACTCCCATTCTTCCACCTTTG-3'
	Reverse primer	5'-AATATGGCTACAGCAACAGGG-3'

TNF-α: Tumor necrosis factor-alpha; IL: Interleukin; PXR: Pregnane X receptor; CAR: Constitutive androstane receptor; GR: Glucocorticoid receptor; CYP: Cytochrome P450.

following reaction conditions: an initial heating cycle of 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 25 s, primer annealing at 60°C for 25 s, and extension at 72°C for 20 s. Melt curves clarified the identity of amplicons, and the housekeeping gene *Gapdh* served as an internal control. The relative mRNA expression level of the target genes was calculated by the comparative Ct (threshold cycle) method, normalized to *Gapdh* mRNA in the same sample. Specific ΔCt was calculated as follows: $\Delta Ct = (Ct_{GAPDH}) - (Ct_{target})$; relative expression was defined as $2^{-\Delta Ct}$.

Statistical analysis

Numerical data were presented as the mean ± standard error (SE). One-way analysis of variance (ANOVA) was performed to determine the statistical significance of differences among the groups, and Student's *t*-test was implemented to verify the statistical significance between two arbitrary groups. Differences at $P < 0.05$ were considered statistically significant.

Results

Effect of intermittent hypoxia on the expression of inflammatory cytokines in the liver

In the IH group, the blood-gas assay results revealed that the minimum oxygen saturation (SaO_2) level was 0.863 ± 0.017, and the arterial partial pressure of oxygen (PaO_2) was 50.00 ± 2.64 mmHg (1 mmHg = 0.133 kPa; data not shown). The SaO_2 and PaO_2 periodically returned from below-normal to normal levels (data not shown). These findings indicated that the IH model was successfully established in the rat. The hepatic lesions and biochemical data from the two rat groups were analyzed. After IH exposure for 14 weeks, H and E-stained tissue sections of the liver were obtained and analyzed, and the mRNA expression levels of inflammatory cytokines were measured as liver injury markers. In the IH group, there were inflammatory cell infiltrates in the portal area, and steatosis of hepatocytes, and the lightly stained cytoplasm of liver cells implied a loose cytoplasm [Figure 1a and 1b]. The hepatic mRNA expression level of IL-1β in the IH group was significantly higher than that in the control group (0.005 ± 0.001 vs. 0.038 ± 0.008, $P = 0.042$) [Figure 1c]. However, TNF-α and IL-6 in the IH group were similar to those in the control group [Figure 1d and 1e]. The above-mentioned findings indicated that hepatic lesions and early-phase inflammation had occurred in the IH group.

Messenger RNA expression levels of cytochrome P450 in the liver

The mRNA expression levels of various CYP molecular species in the liver were also assessed in the rats of the two groups. The mRNA expression level of *Cyp1a2* was significantly higher in the IH-exposed rats than in the control rats (0.022 ± 0.002 vs. 0.0050 ± 0.0002, $P = 0.029$) [Figure 2a]. Moreover, a trend of decreasing hepatic *Cyp2c9*, *Cyp2c19*, *Cyp2d4*, and *Cyp3a2* levels was observed in the IH group [Figure 2b-2e]. These findings were consistent with the finding in previous studies that inflammation may induce the downregulation of CYPs.[12,13]

Messenger RNA expression level of nuclear factor-κB in the liver

Contrary to the enhanced secretion of proinflammatory cytokines, nuclear translocation of the transcription factor NF-κB was reduced in the liver of IH-exposed rats, but the difference relative to the control was not statistically significant [Figure 3a].

Messenger RNA expression levels of pregnane X receptor, constitutive androstane receptor, and glucocorticoid receptor in the liver

Cyp3a expression has been shown to be regulated by nuclear receptors, including PXR and CAR,[14-16] where with a decrease in nuclear translocation of these receptors, the expression of Cyp3a also declined. In addition, the synthesis and nuclear translocation of these receptors correlated negatively with NF-κB nuclear translocation.[17,18] In our experiment, we found that the hepatic mRNA expression levels of *PXR* and *CAR* remained unchanged in the IH group compared with that in the control group [Figure 3b and 3c]. However, GR expression was obviously diminished in the IH group (0.056 ± 0.012 vs. 0.032 ± 0.005, $P = 0.035$) [Figure 3d]. These results demonstrated that the reduced hepatic expression of CYPs observed in the IH-exposed rats may be attributed to the lowered synthesis and nuclear translocation of GR.

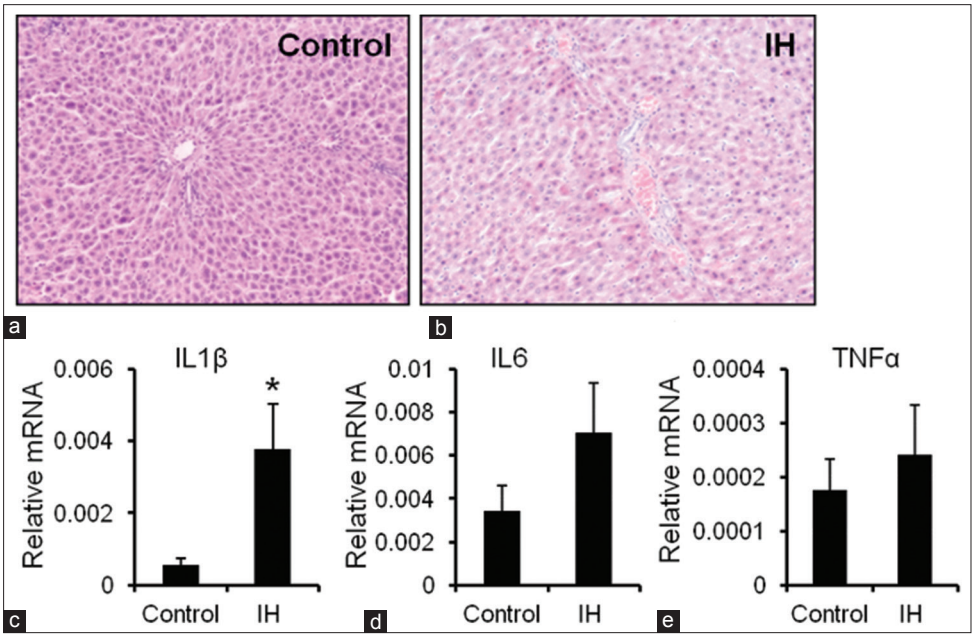

Figure 1: Effect of IH on the expression of inflammatory cytokines in the liver (H and E, original magnification, ×100). (a and b) H and E staining of liver tissues harvested from the control and IH groups. (c-e) The mRNA expression levels of *IL-1β, IL-6*, and *TNF-α* were determined in the control and IH groups by quantitative reverse transcription polymerase chain reaction, using *Gapdh* as the housekeeping gene. The data are presented as the mean ± standard error. *$P < 0.05$, compared with the control group. IH: Intermittent hypoxia; mRNA: messenger RNA; IL-1β: Interleukin-1beta; TNF-α: Tumor necrosis factor-alpha.

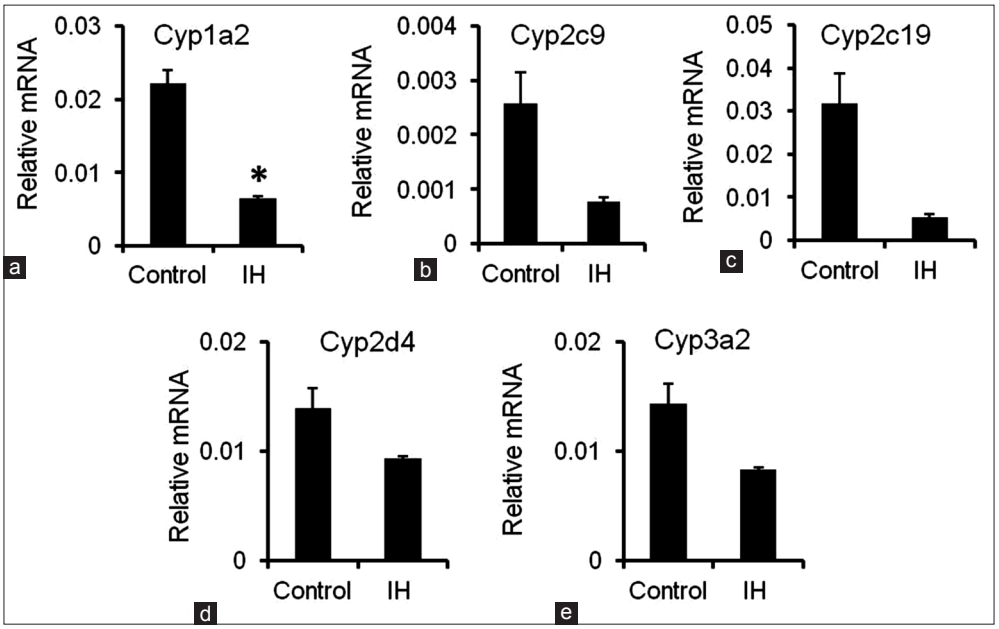

Figure 2: mRNA expression levels of CYPs in the liver. (a-e) The mRNA expression levels of *Cyp1a2*, *Cyp2c9*, *Cyp2c19*, *Cyp2d4*, and *Cyp3a2* were measured by quantitative reverse transcription polymerase chain reaction. *Gapdh* was used as the housekeeping gene. The data are presented as the mean ± standard error. *$P < 0.05$, compared with the control group. IH: Intermittent hypoxia; mRNA: messenger RNA; CYPs: Cytochrome P450.

Discussion

OSA is a common sleep disorder in which complete or partial airway obstruction occurs and is caused by pharyngeal collapse during sleep. The condition results in loud snoring or choking, frequent awakenings, disrupted sleep, and excessive daytime sleepiness. The repeated pauses in breathing can lead to IH and increased ROS.[19] In 1997, Henrion *et al*.[20] first reported two cases of hepatitis combined with the hypoxia of OSA and associated the OSA syndrome with liver damage. In 2005, Tanné *et al*.[3] reported that the hepatic damage ratio was higher in patients with OSA, which was confirmed by liver biopsy, and included increased transaminase, steatohepatitis, liver cell necrosis, and liver fibrosis. Savransky *et al*.[4] researched chronic IH-associated liver disorders using animal experiments, revealing that the chronic IH of OSA could lead to liver

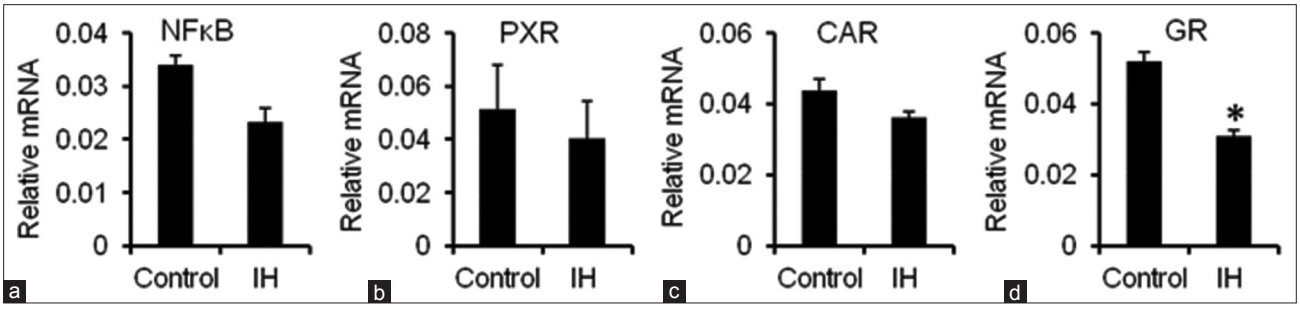

Figure 3: mRNA expression levels of *NF-κB, PXR, CAR*, and *GR* in the liver. (a-d) The mRNA expression levels of *NF-κB, PXR, CAR*, and *GR* were measured by quantitative reverse transcription polymerase chain reaction. *Gapdh* was used as the housekeeping gene. The data are presented as the mean ± standard error. *$P < 0.05$, compared with the control group. IH: Intermittent hypoxia; mRNA: Messenger RNA; PXR: Pregnane X receptor; CAR: Constitutive androstane receptor; GR: Glucocorticoid receptor.

damage. In our study, we adopted an animal model of IH and showed meaningful liver damage in the rats, which is consistent with the above studies.

IH caused by anoxia and reoxygenation can activate different inflammatory responses, inducing the release of proinflammatory cytokines, chemokines, and adhesion molecules, which lead to endothelial injury and dysfunction.[21] It is considered that the inflammatory responses are associated with OSA-related extrapulmonary complications.[22] Hence, determination of the important proinflammatory cytokines (i.e., IL-1β, IL-6, and TNF-α) of the liver can reflect the state of inflammation damage to a certain extent. In the IH group of our study, the hepatic mRNA level of IL-1β was higher than that in the control group. IL-1β may be the key factor promoting the inflammation. In contrast, another study did not find statistically significant differences in the serum levels of IL-1β between OSA patients and a control group,[23] and considered that IL-1β had no close relationship with OSA and its complications. Therefore, more animal and human studies are needed to determine the exact relationship between this proinflammatory cytokine and OSA.

In animal studies, it has been found that IH-induced liver damage affects the rate of drug metabolism, such as that of theophylline, which is widely used for treating respiratory system diseases.[24] In humans, the CYP1, CYP2, and CYP3 families are involved in hepatic drug metabolism. Among these family members, CYP1A2, CYP2C9, CYP2C8, CYP2E1, and CYP3A4 are expressed abundantly in the liver.[25] In fact, CYP levels influence the liver capability of drug metabolism to a large extent. Moreover, it was reported that CYP expression is regulated by the hypoxic state, as it was found that the expression of CYP2J2 was downregulated in HepG2 cells after 48 h of hypoxic exposure.[26] In animal experiments, CYP1A1 and CYP1A2 protein expression was decreased in the liver after 24 h of hypoxic exposure, whereas expression of the CYP3A6 protein was increased.[18] Although the effects of hypoxemia on the expression of CYPs depend on the cell type, IH obviously causes a general decrease in the expression levels in the liver,[24] which is consistent with our findings.

In this study, *Cyp1a2* mRNA expression decreased in the IH group. This implies that the IH caused by OSA can damage liver metabolism. Such decline of hepatic metabolic capability would translate into a reduction of drug clearance and removal in the serum. When the liver is unable to metabolize the drug, the chemical will accumulate and create excessive toxic effects in the organ, thereby increasing the liver damage. As the center of the body's metabolic activity, liver function damage translates into decreased metabolic function. Therefore, close attention must be paid to the liver function of patients on medication, especially those on drugs that are metabolized hepatically.

Although there is no clear explanation as to the exact mechanism of how IH influences CYP expression, it has been reported that inflammatory factors may have an influence on the transcription of CYPs.[18] As shown in our study, the increased expression of IL-1β may inhibit the transcription of CYPs and cause a decrease in their expression.

PXR and CAR regulate the drug-metabolizing enzymes, including the phase I metabolic enzymes CYP3A4, CYP7A1, CYP2B6, CYP2C9, CYP2C18, and CYP2C19. PXR and CAR pathways can regulate the expression of CYP2C9,[27] and metabolic enzymes such as CYP2C18 and CYP2C19 mainly play increased roles. Our experiments showed that the mRNA expression level of *GR* was reduced in the IH group, which further explains the corresponding decreased expression of *Cyp1a2*, and possible *Cyp2c9*, *Cyp2c19*, and *Cyp3a2* in the rat model.

In conclusion, the present study examined the effects of IH on the liver. We found that IH alters the expression of inflammatory cytokines and CYPs in the liver, which may contribute to the alteration of hepatic drug metabolism in patients with OSA. Therefore, it is important to pay close attention to the liver function of patients with OSA, especially if they are being medicated with drugs that are metabolized hepatically.

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Conflicts of interest

There are no conflicts of interest.

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