

Altered gene expression of hepatic cytochrome P450 in a rat model of intermittent hypoxia with emphysema

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Abstract. Patients with respiratory overlap syndrome (OS), defined as concomitant chronic obstructive pulmonary disease and obstructive sleep apnea syndrome, may exhibit an increased blood concentration of ingested drugs. This poor elimination of drugs is primarily attributed to downregulated gene expression of the drug-metabolizing cytochrome P450 enzymes (CYPs) in the liver. However, the underlying mechanisms of the decreased expression of CYPs in OS are poorly understood. In order to address this, a rat model of intermittent hypoxia with emphysema was evaluated in the present study, by analyzing liver gene expression using the reverse transcription-quantitative polymerase chain reaction. Intermittent hypoxia and cigarette smoke exposure caused upregulation of hepatic inflammatory cytokines, while CYPs were downregulated. This downregulation of CYPs was associated with an increase in nuclear factor (NF)- κ B expression and a decrease in the expression of nuclear receptors pregnane X receptor, constitutive androstane receptor and glucocorticoid receptor, which are the upstream regulatory molecules of CYPs. The results of the present study indicated that, during the development of OS, systematic inflammatory reactions may downregulate hepatic CYP gene expression via the NF- κ B signaling pathway.

Introduction

Chronic obstructive pulmonary disease (COPD) and obstructive sleep apnea syndrome (OSAS) represent two of the most prevalent chronic respiratory disorders in clinical practice. Respiratory overlap syndrome (OS), defined as the coexistence of these two diseases, occurs in ~1% of adults and leads to the development of increased nocturnal oxygen desaturation compared with mono-COPD or OSAS (1-3). The possible coexistence of COPD with OSAS is notable, as systemic inflammation develops in each disorder (3,4) and may contribute to the pathogenesis of associated comorbidities. Hypoxia induces systemic inflammatory reactions and acts as an aggravating factor of liver injury (5,6). It has been hypothesized that, in patients with mono-COPD or OSAS, inflammatory mediators generated in the lung may 'spill over' into the bloodstream and promote the release of inflammatory proteins from the liver, which may cause damage to target organs and act as a source of systemic inflammation (7,8).

Inflammation modulates the expression of hepatic metabolizing enzymes. Cytochrome p450 enzymes (CYPs) catalyze the oxidative metabolism of numerous drugs, and the level of CYP expression may affect drug efficacy, toxicity and consequently, therapeutic outcome (9). Previous studies demonstrated that the blood concentration of theophylline was above the normal range when the expression and activity of CYP1A2 was inhibited (10,11). A further previous study demonstrated that sleep hypoxia combined with emphysema synergistically enhanced hepatic inflammation and produced a more apparent liver-derived inflammatory state (12). It has been reported that inflammatory challenges may suppress the expression of major CYPs (13). Underexpression of the nuclear receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR), as upstream regulatory molecules, may inhibit the transcription of CYPs (11-13) by attenuating nuclear translocation (14,15). It is hypothesized that severe inflammation in the liver tissues of patients with OS alters hepatic metabolism via nuclear receptors.

In the present study, a previously-published rat model of OS was developed by exposing rats to intermittent hypoxia (IH) and cigarette smoke (CS). Gene expression analysis of liver samples with reverse transcription-quantitative polymerase

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chain reaction (RT-qPCR) was used to evaluate the effect of IH with CS on inflammatory cytokines and CYPs. The present study may provide a molecular mechanism to explain adverse drug reactions in patients with OS.

Materials and methods

Ethics statement. Rats were used in accordance with the protocol approved by the Animal Care Committee of Tianjin Medical University (permit no. 2010-0002).

Animals and treatments. Rats were provided by the Model Animal Center of the Radiological Medicine Research Institute, Chinese Academy of Medical Science (Beijing, China), and housed in standard laboratory cages (5 rats/cage) with food and water available *ad libitum*. As described previously (12), a total of 30 male Wistar rats weighing 180 ± 20 g at age 6 weeks were divided into two groups of 15 according to exposure condition, as follows: i) Control group; ii) IH with CS experimental group. For IH, the rats were treated in a 120 sec cycle, comprising 30 sec nitrogen followed by 90 sec air, between 9:00 a.m. and 5:00 p.m. daily. For CS exposure, the rats underwent whole-body exposure to the smoke of five unfiltered cigarettes (Daqianmen, Yunnan, China; ≤ 15 mg tar, ≤ 1.1 mg nicotine and ≤ 13 mg CO) for 30 min twice-daily (before 9:00 a.m. and after 5:00 p.m.), 7 days/week for 14 weeks, inside a 0.6 m^3 custom-made plexi-glas chamber (16,17).

Measurement of serum liver enzymes. Blood samples from control and experimental rats were centrifuged at 600 g for 15 min at 4°C , and the serum was stored at -80°C prior to being assayed. The serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentrations were quantified using a transaminase CII test, according to the manufacturer's protocol (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Liver tissue sampling. Following treatment, 10 rats from the control and experimental groups were anesthetized with 10% chloral hydrate ($0.3 \text{ ml}/100 \text{ g}$ body weight) and sacrificed. For gene expression analysis using reverse transcription-quantitative polymerase chain reaction (RT-qPCR), liver tissues were excised, rinsed in ice-cold PBS, frozen in liquid nitrogen and stored at -80°C prior to analysis. For hematoxylin and eosin staining, liver tissues were fixed in 10% formalin at 4°C overnight, embedded in paraffin, and sliced into $5\text{-}\mu\text{m}$ -thick sections. Sections were then stained with 1% hematoxylin and eosin solution for 5 min at room temperature, according to the manufacturer's protocol.

Preparation of RNA from tissue samples. RNA was extracted from liver tissues using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The extract yield and quality were determined by measuring the absorbance at 260 and 280 nm using the Maestro Nano Micro-Volume Spectrophotometer (Maestro, Inc., Hsinchu, Taiwan). The absorbance ratio at 260:280 nm was between 1.8 and 2.0. The RNA was subsequently reverse-transcribed into cDNA.

RT-qPCR. mRNA ($3 \mu\text{g}$) was reverse-transcribed into cDNA using oligo(dT) primers for 1 h at 50°C , with the TIAN Script RT kit (Tiangen Biotech Co., Ltd., Beijing, China), according to the manufacturer's protocol. The cDNA served as a template for qPCR, which was performed using SYBR Green PCR core reagents (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Specific gene primers were designed using the PrimerQuest SM software (<http://sg.idtdna.com/Primerquest/Home/Index>; Integrated DNA Technologies, Inc., Coralville, IA, USA) and commercially produced (BGI Tech, Shenzhen, China) (Table I). DNA amplification was performed using a CFX96 Real-Time System (Bio-Rad Laboratories, Inc.) with the following reaction conditions: An initial heating cycle of 95°C for 2 min; 40 cycles, alternating between denaturation at 95°C for 25 sec and primer annealing at 60°C for 25 sec; and final extension at 72°C for 20 sec. Melt curves were used to clarify the identity of the amplicons and the housekeeping gene GAPDH served as an internal control. The relative mRNA expression of targeted genes was calculated using the comparative C_q (threshold cycle) method and normalized to GAPDH mRNA in the same sample (18). The specific ΔC_q was calculated as follows: $[\Delta C_q = (C_{q\text{GAPDH}}) - (C_{q\text{target}})]$; relative expression was defined as $2^{-\Delta\Delta C_q}$.

Statistical analysis. The numerical data are presented as the mean \pm standard error of the mean. The statistical significance of the differences between the two groups was assessed using Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference. Statistical analysis was performed using Microsoft Excel software version 2007 (Microsoft Corporation, Redmond, WA, USA).

Results

IH with CS exposure causes elevated expression of liver enzymes, upregulated mRNA expression of inflammatory cytokines and hepatocyte damage. Consistent with a previous study (12), IH with CS exposure resulted in apparent emphysemic alterations in rat lungs and decreased blood gas concentration, indicating that the rat model of IH with emphysema was successfully established (data not presented). Hematoxylin and eosin staining of the liver demonstrated that various hepatic lesions were observed in the IH with CS group, and not in the control group (Fig. 1A and B). Compared with the control group, hepatic lobules in the IH with CS group exhibited partial clarity and integrity, sinusoids were broadened, inflammatory cell infiltration was observed in the periportal space and various foci of lobular inflammatory cell accumulation were noted. Inflammatory cell infiltrates were observed in the portal area and light staining of the cytoplasm of liver cells suggested cytoplasmic loss (Fig. 1C).

The concentration of serum ALT and AST in the experimental group was increased compared with the control group ($P < 0.05$; Table II), which suggested a more severe impairment of hepatocyte function. Additionally, the mRNA expression levels of interleukin (IL)- 1β , IL-6, and tumor necrosis factor (TNF)- α in the livers of the experimental rats were significantly increased compared with the control group ($P < 0.05$; Fig. 2). The results of the present study suggested that early-phase inflammation and mild hepatocyte damage had occurred.

Table I. Primer sequences for the present study.

Genes	Forward (5'-3')	Reverse (5'-3')
IL-1 β	TCCCTGAACTCAACTGTGAAATA	GGCTTGGAAGCAATCCTTAATC
IL-6	GAAGTTAGAGTCACAGAAGGAGTG	GTTTGCCGAGTAGACCTCATAG
TNF- α	ACCTTATCTACTCCCAGGTTCT	GGCTGACTTTCTCCTGGTATG
NF- κ B	AGACATCCTTCCGCAAATC	TAGGTCCATCCTGCCCATAA
PXR	GAAGATCATGGCTGTCCTCAC	CGTCCGTGCTGCTGAATAA
CAR	GAGACCATGACCAGTGAAGAAG	AGTCAGGGCATGGAAATGATAG
GR	CAGCAGTCAAATGGGCAAAG	GGGCAAATGCCATGAGAAAC
CYP1A2	GACAAGACCCTGAGTGAGAAG	GAGGATGGCTAAGAAGAGGAAG
CYP2C9	CCCAAGGGCACAACCATATTA	CTTTCTGGATGAAGGTGGCA
CYP2C19	CCCAAGGGCACAACCATATTA	TTTGACCCTCGTCACTTTCTG
CYP2D4	CCTTTCAGCCCTAACACTCTAC	ATGAAGCGTGGGTCATTGT
CYP3A2	GGAAACCCGTCTGGATTCTAAG	GAAGTGTCTCATAAAGCCCTGT
GAPDH	ACTCCCATCTCTCCACCTTTG	AATATGGCTACAGCAACAGGG

IL, interleukin; TNF, tumor necrosis factor; NF, nuclear factor; PXR, pregnane X receptor; CAR, constitutive androstane receptor; GR, glucocorticoid receptor; CYP, cytochrome P450.

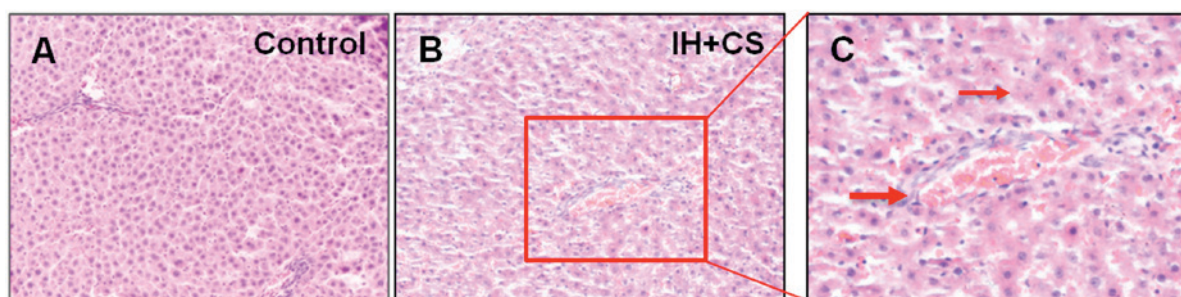


Figure 1. Liver pathology of rat exposed to IH and CS. Control rats or rats exposed to IH+CS were sacrificed and liver tissues were processed to conduct hematoxylin and eosin staining. Compared with the integrity and clarity of the liver tissues in (A) the control group, the liver tissues from (B) the IH+CS group demonstrated hepatocytes which were arranged in rows around central veins, and were visible as polygons in the sinusoid; magnification, $\times 200$. (C) Further magnification of a section of IH+CS liver tissue; magnification, $\times 400$. IH, intermittent hypoxia; CS, cigarette smoke.

Underexpressed mRNA levels of CYPs in the injured liver. Expression of each CYP is influenced by a unique combination of mechanisms and factors, including genetic polymorphisms, xenobiotic induction, regulation by cytokines, hormones and disease states, in addition to sex, age, and others (13). Compared with the control group, the IH with CS group exhibited markedly increased mRNA expression of CYP1A2, CYP2C9, CYP2C19, CYP2D4, and CYP3A2 (Fig. 3). The results of the present study demonstrated that liver inflammation and hepatocyte damage affect the transcription of major CYPs in a rat model of IH with emphysema, which was consistent with the previous literature suggesting inflammation may induce downregulation of CYP expression (19,20).

Upregulation of nuclear factor (NF)- κ B and subsequent down-regulation of nuclear receptors in the liver. Transcription factor NF- κ B serves a role in inflammatory reactions and oxidative stress (21,22). CYP3A expression is modulated by nuclear receptors, including PXR and CAR (23-25). If the nuclear translocation of these receptors is decreased, CYP3A expression will

consequently decrease. In addition, the synthesis and nuclear translocation of these receptors is negatively-associated with NF- κ B nuclear translocation (14,26). Consequently, NF- κ B expression was significantly increased in the IH with CS rats, compared with the control group ($P < 0.05$; Fig. 4). Additionally, in the IH with CS group, the hepatic mRNA expression of PXR, CAR and the glucocorticoid receptor (GR) was significantly decreased compared with that in the control group ($P < 0.05$; Fig. 5). The results of the present study demonstrated that upregulated NF- κ B may be involved in reduced hepatic CYP expression, by negatively impacting the synthesis and nuclear translocation of PXR, CAR and GR.

Discussion

The emerging pathophysiology of OS encompasses intermittent hypoxia and emphysema (27). In the present study, a rat model of intermittent hypoxia with emphysema was developed. The results of the present study suggested that an early phase of inflammation and mild hepatocyte damage had occurred in

Table II. Serum ALT and AST levels in the liver.

Group	n	ALT, U/l	AST, U/l
Contro	15	21.0±5.3	17.8±3.0
IH+CS	15	50.5±2.1 ^a	26.0±3.2 ^a

^aP<0.05. IH, intermittent hypoxia; CS, cigarette smoke; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

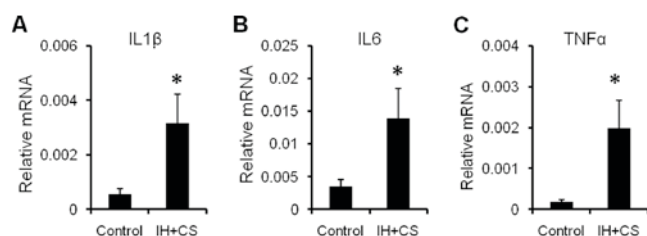


Figure 2. mRNA expression levels of inflammatory cytokines in the liver. The mRNA expression levels of the inflammatory cytokines (A) IL-1 β , (B) IL-6, and (C) TNF- α in the liver were measured using the reverse transcription-quantitative polymerase chain reaction. GAPDH was used as an internal reference. The data are presented as the mean \pm standard error of the mean, and were obtained from 10 rats/group. Student's t-test; *P<0.05 vs. the control. IL, interleukin; TNF, tumor necrosis factor; IH, intermittent hypoxia.

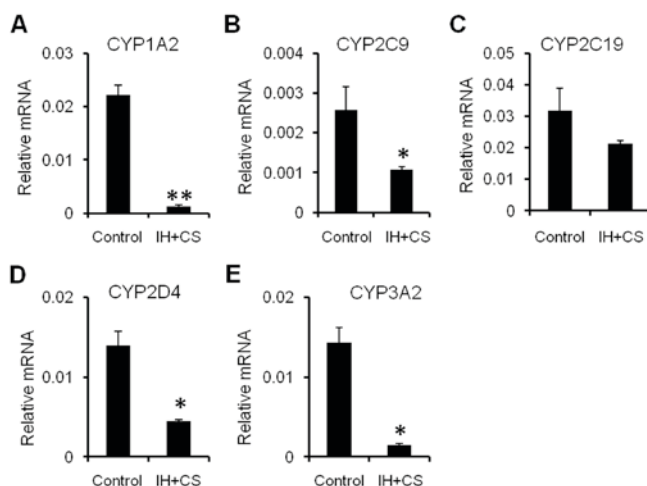


Figure 3. mRNA expression levels of CYPs in the liver. The mRNA expression levels of (A) CYP1A2, (B) CYP2C9, (C) CYP2C19, (D) CYP2D4 and (E) CYP3A2 were measured using the reverse transcription-quantitative polymerase chain reaction. GAPDH was used as an internal reference. The data are presented as the mean \pm standard error of the mean, and were obtained from 10 rats/group. Student's t-test; *P<0.05, **P<0.01 vs. the control. CYP, cytochrome P450; IH, intermittent hypoxia.

the liver. The results of the present study were consistent with previous studies, which stated that hypoxia contributes to liver injury (28,29). Alterations in hepatic CYP expression, and the mechanisms underlying these alterations, were analyzed in the present study using the IH with CS rat model. In the IH with CS group, the hepatic mRNA expression levels of a number of CYP molecular species, specifically CYP1A2, CYP2C9, CYP2C19, CYP2D4 and CYP3A2, were markedly decreased compared with the control group.

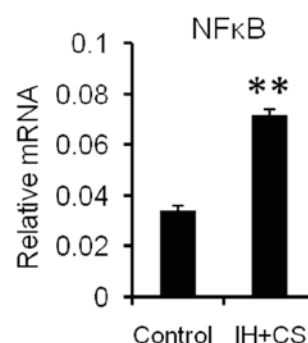


Figure 4. mRNA expression level of NF- κ B in the liver. Following the establishment of the rat model, the liver was removed and the mRNA expression level of NF- κ B was measured using the reverse transcription-quantitative polymerase chain reaction. GAPDH was used as an internal reference. The data are presented as the mean \pm standard error of the mean, and were obtained from ten rats per group. Student's t-test; **P<0.01 vs. the control. NF- κ B, nuclear factor- κ B; IH, intermittent hypoxia.

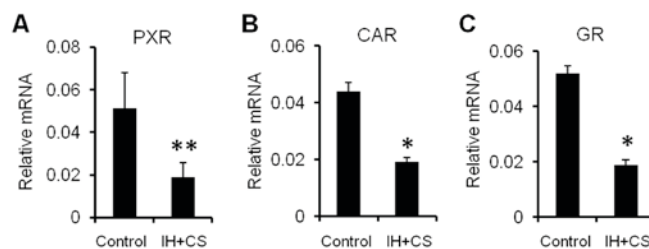


Figure 5. mRNA expression levels of (A) PXR, (B) CAR, and (C) GR in the liver. After established successfully model, the liver was removed, and the mRNA expression levels of PXR, CAR, and GR were measured using the reverse transcription-quantitative polymerase chain reaction. GAPDH was used as an internal reference. The data are presented as the mean \pm standard error of the mean, and were obtained from ten rats per group. Student's t-test; *P<0.05, **P<0.01 vs. the control. PXR, pregnane X receptor; CAR, constitutive androstane receptor; GR, glucocorticoid receptor; IH, intermittent hypoxia.

The present study sought to analyze why the expression of hepatic CYPs decreased in the rat model of intermittent hypoxia with emphysema. It is known that CYPs are generated in damaged hepatocytes, where the degree of damage may affect the quantity and quality of CYPs. Previous studies have demonstrated that the severity of liver injury is positively-associated with levels of hypoxia and inflammation (30,31). Compared with patients with only COPD or OSAS, nocturnal hypoxemia, hypoxia and hypercapnia are more severe in patients with OS (32), and the present study demonstrated that intermittent hypoxia and emphysema interact synergistically. The mechanism of the decrease in CYP expression was analyzed by focusing on hepatic inflammation, as the pathological conditions of early-phase hepatic inflammation and mild hepatocyte damage were detected in the present experimental model. Hepatic inflammation is promoted by the binding of cyclic pro-inflammatory factors to Toll-like receptor 4 in hepatic Kupffer cells. The activated Kupffer cells subsequently initiate the secretion of inflammatory cytokines, which promote the activity of NF- κ B (21). Activated NF- κ B dissociates from the inhibitor of NF- κ B and translocates to the nucleus (33). Nuclear NF- κ B forms a complex with the transcription factor GR and prevents the binding of GR to

GR-responsive elements, thereby inhibiting the transcription of PXR and CAR (14,15). The decrease in PXR and CAR expression causes a decrease in nuclear translocations (26,34). Additionally, the decrease in the expression of PXR and CAR, which bind to the responsive elements of DNA, results in the inhibition of CYP3A transcription and a subsequent decrease in its expression (35-37). Hepatic NF- κ B nuclear translocation was examined in the present study, and was observed to be increased in the experimental group compared with the control group. By contrast, the hepatic mRNA expression of PXR, CAR and GR was decreased in the experimental group. The results of the present study demonstrated that, in a rat model of intermittent hypoxia with emphysema, hepatic inflammatory cytokines activate NF- κ B, which inhibits the transcription of PXR and CAR. This inhibited transcription subsequently leads to a decrease in nuclear translocation, which inhibits the transcription of CYP3A.

The transcription of CYP2C is modified by CAR (38,39). Therefore, it is hypothesized that the mechanism underlying the decrease in the expression level of CYP2C is identical to that underlying the decrease in the CYP3A expression level. However, the transcription of CYP1A and CYP2D is not primarily regulated by PXR and CAR (40,41). Therefore, the decrease in the nuclear translocation of these CYP molecular species cannot be explained by the decrease in the nuclear translocation of PXR and CAR. However, it has been previously demonstrated that the expression levels of these CYP molecular species are downregulated by inflammatory cytokines (42). It is therefore hypothesized that the decrease in the hepatic expression levels of CYP1A and CYP2D in the IH with CS group, in the present study, may be triggered by inflammatory cytokines, as is the case with other CYP molecular species.

In conclusion, early-phase hepatic inflammation and mild hepatocyte damage was detected in the rat model of intermittent hypoxia with emphysema generated in the present study; additionally, hepatic CYP expression was markedly decreased. Based on the results of the present study, it is hypothesized that, during the development of OS, systematic inflammatory reactions may downregulate hepatic CYP gene expression via the NF- κ B signaling pathway. The results of the present study may provide an important molecular mechanism to explain adverse drug reactions in patients with OS.

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