Evaluation of hepatic metabolism and pharmacokinetics of ibuprofen in rats under chronic hypobaric hypoxia for targeted therapy at high altitude

Shefali Gola\textsuperscript{a}, Asheesh Gupta\textsuperscript{a,∗}, Gaurav K. Keshri\textsuperscript{a}, Madhu Nath\textsuperscript{b}, Thirumurthy Velpandian\textsuperscript{b}

\textsuperscript{a} Department of Pharmacology, Defence Institute of Physiology and Allied Sciences (DIPAS), DRDO, Lucknow Road, Timarpur, Delhi 110054, India

\textsuperscript{b} Pharmacokinetic Laboratory, Department of Pharmacology, All India Institute of Medical Sciences (AIIMS), New Delhi 110029, India

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With studies indicative of altered drug metabolism and pharmacokinetics (DMPK) under high altitude (HA)-induced hypobaric hypoxia, consideration of better therapeutic approaches has continuously been aimed in research for HA related illness management. DMPK of drugs like ibuprofen may get affected under hypoxia which establishes the requirement of different therapeutic dose regimen to ensure safe and effective therapy at HA. This study examined the effects of the chronic hypobaric hypoxia (CHH) on hepatic DMPK of ibuprofen in rats. Experimental animals were exposed to simulated altitude of 7620 m (~25,000 ft) for CHH exposure (7 or 14 days) in decompression chamber and administered with ibuprofen (80 mg/kg, body weight, p.o.). Results demonstrated that CHH significantly altered PK variables of ibuprofen and activities of both phase-I and II hepatic metabolic enzymes as compared to the animals under normoxic conditions. Hepatic histopathological observations also revealed marked alterations. Increase in pro-inflammatory cytokines/chemokines viz. IL-1β, IL-2, IFN-γ, TNF-α exhibited close relevance with diminished CYP2C9 expression under CHH. Moreover, the down-regulated CYP2C9 level further supported the underlying mechanism for reduced metabolism of ibuprofen and as a result, increased retention of parent drug in the system. Increased mean retention time, Vd, ½ of ibuprofen, and decreased AUC, Cmax and clearance during CHH further strengthened the present findings. In conclusion, CHH exposure significantly affects hepatic DMPK of ibuprofen, which may further influence the usual therapeutic dose-regimen. Further, there is requirement of human studies to evaluate their susceptibility toward hypobaric hypoxia.

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1. Introduction

An intricate sequence of pathophysiological effects arises in human body following exposure to high altitude (HA). At HA, the first and foremost environmental stress i.e., hypobaric hypoxia exerts impact on the human body, causing a wide range of physiologic illnesses such as acute mountain sickness (AMS), high altitude cerebral edema (HACE) and high altitude pulmonary edema (HAPE) [1]. Hypobaric hypoxia affects the normal physiology and functions of vital organs like lungs, brain, heart and liver due to subnormal oxygen concentration in the cells [2]. Liver regulates various biochemical pathways including the metabolism of endogenous, exogenous compounds and their detoxification. Hepatic metabolism is a complex physiological process aiming at converting the parent lipophilic drug to a more polar metabolite. Oxygen plays a crucial role of substrate for drug oxidations as a terminal electron acceptor in the mitochondria that regulates other processes dependent upon the cellular redox state and synthesis of essential high energy co-factors and reducing equivalents (ATP and NAD) required for drug transport and conjugation pathways. The enzymes of these metabolic pathways possess different affinities for oxygen, thus their functional sensitivity to the severity of hypoxia also differs. Therefore, for regulation of these metabolic pathways, liver requires more oxygen than other tissues and is more prone to hypoxia-induced oxidative stress [3].

Previous studies have reported alterations in metabolism of drugs under hypoxia [4,5]. Various studies on drug metabolism (DM) and pharmacokinetics (PK) of common drugs like car-
bolic anhydride inhibitors (acetazolamide), anti- pyretic and anti-angolic (acetaminophen), corticosteroids (dexamethasone, prednisolone), non-steroidal anti-inflammatory drugs (NSAIDs; ibuprofen), furosemide and lithium etc., have demonstrated alterations in oxidative as well as conjugation pathways along with elimination of these drugs [6-8]. Therefore, it is crucial to understand the requirement of safe therapy under HA-induced hypoxia for treating various HA-related maladies. For the use of NSAIDs as prophylactic and therapeutic intervention under HA-induced headache, the focus has been continuously aimed on their safe use, effectiveness and absorption, distribution, metabolism and elimination (ADME) properties. Various studies have been conducted earlier for studying usefulness and efficacy of ibuprofen for treatment of HA-induced headache [9,10]. Previously, our study reported that acute hypobaric hypoxia (24 h) exposure significantly altered DMPK of ibuprofen [7]. However, it necessitates further investigation to evaluate the effect of chronic hypobaric hypoxia (CHH) on DMPK of ibuprofen, for safe and effective administration as a therapeutic intervention.

CYP2C9 (isoform of CYP450) is best known for its significant role in metabolism of ibuprofen. In conditions associated with oxygen tension, the activity and expression of various CYP proteins get affected which implies that their expression and/or activity may be implicated in hypoxic conditions. Earlier studies have revealed altered expression of isoforms of CYP450 such as CYP1A1, CYP1A2, CYP2B6, CYP2C9 and CYP2C19, CYP2E1 under hypoxic conditions [11,12]. However, there is no conclusive evidence to show the role of CHH in affecting hepatic metabolism and CYP2C9 activity and expression. Hence, the aim of the present study was to investigate the effect of CHH stress on hepatic DM, PK of ibuprofen and protein expression levels of CYP2C9 and inflammatory cytokines/chemokines, if any. Furthermore, the probable associated mechanism involved in regulation of activity and expression of CYP-mediated DM of ibuprofen under CHH has also been determined. Thus, the present work has therapeutic implications in case of any altered dose-regimen of ibuprofen under CHH conditions.

### 2. Materials and methods

#### 2.1. Experimental animals

Experiments were conducted on adult male Sprague-Dawley rats (200±20 g; animal colony of DIPAS, Delhi). The normoxic animals were maintained under controlled environment at the institute’s animal house at temperature of 25±1°C, humidity 55±5% and 12 h: 12 h light: dark cycle with free access to food and water. The study was approved by the Institute’s Animal Ethical Committee (IAEC-02/DIPAS/2013), and experiments were performed in accordance with the national guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) of the Government of India.

#### 2.2. Experimental design for PK under CHH

A total of 24 rats were randomized into four groups (six animals in each) and treated with various regimes: group I and III remained at sea level atmospheric pressure within the same laboratory conditions; experimental rats of group II and IV were exposed to CHH for duration of 7 and 14 days respectively in decompression chamber (Seven Star, Delhi, India) at a simulated altitude of 7620 m above sea level (~25000 ft, 41329.9 Pa/310 mm Hg), as described earlier [7]. The decompression chamber was maintained at 28 ± 2°C, 55 ± 5% humidity and air flow 9–10 l/min during the exposure to prevent accumulation of exhaled gases. Single dose of ibuprofen (80 mg/kg body weight, p.o.) prepared in 2% methylcellulose vehicle was administered immediately after 7 or 14 days CHH exposure to all normoxic and hypoxic groups, as described earlier [7]. Ibuprofen was administered after stipulated exposure duration because pressure in the decompression chamber cannot be released repeatedly during hypobaric hypoxia exposure. The blood samples were collected through orbital sinus at 0 min time point for pre-dose and at 0.25, 0.5, 1, 2, 3, 4 and 6 h for post-dose sample for each hypoxia exposure period. The blood samples were immediately centrifuged at 419 g for 10 min and the plasma was separated and stored at −80°C for further analysis by liquid chromatography–mass spectrometry (LC–MS/MS).

#### 2.2.1. Evaluation of PK variables by LC–MS/MS

The plasma concentration of ibuprofen was analyzed using LC–MS/MS bioanalytical method, carried out as described previously [13]. The LC chromatographic separation system (Thermo Scientific, Waltham, MA, USA) consists of a quaternary pump (Agilent 1100, Agilent Technologies, Santa Clara, CA, USA), an online solvent degasser and an auto-sampler. An Applied Biosystems/MSD Sciex API 4000 triple quadrupole mass spectrometer (MDS Sciex, CA, USA) equipped with ESI source was used for mass spectrometric analysis and detection. Instrument control and data acquisition were carried out with Applied Biosystems/MSD Sciex Analyst software (version 1.4). The LC–MS/MS analysis was performed at room temperature using a Chromolith Flash RP-18 endcapped column (25 mm × 4.6 mm) (Merck Life Science Pvt., Ltd., Mumbai, Maharashtra, India). Acetonitrile/water and methanol/water mixtures were initially evaluated for use as mobile phase. Analytes were eluted with mobile phase A, containing water with 0.3% formic acid and, mobile phase B containing acetonitrile with 0.3% formic acid (35:65, v/v) pumped at a flow rate of 500 μl/min.

#### 2.2.2. Sample preparation

20 μl of plasma sample was mixed with 180 μl of extraction solvent containing 70% acetonitrile with 0.1% formic acid and 250 ng/ml sulphamidomethione and vortexed for 1 min. The mixture was centrifuged for 10 min at 10,000 rpm and the resulting supernatant was collected and subjected for LC–MS/MS analysis. The concentration data obtained was analyzed through WinNonlin pharmacokinetic software Version 5.1, Scientific Consultants, MD, USA for further evaluation of PK variables viz. elimination half-life (T1/2), mean residence time (MRT), clearance (CL) and volume of distribution (Vd), Cmax, Tmax, AUC (obs) 0-t etc.

#### 2.3. Experimental design for hepatic drug metabolism under CHH

In another set of experiment, forty animals were randomly allocated into four groups. Animals of the group I and III remained at sea level atmospheric pressure within the same laboratory conditions and were administered with ibuprofen (80 mg/kg body weight, p.o.) daily for duration of 7 and 14 days respectively. Animals of group II and IV were exposed in decompression chamber for duration of 7 and 14 days respectively and were also administered with ibuprofen (80 mg/kg body weight, p.o.) daily for stipulated period of hypoxia exposure.

#### 2.3.1. Hepatic drug metabolizing (phase I and II) enzyme activities

After CHH exposure (7 or 14 days), all the rats in the normoxic and hypoxic groups were sacrificed and microsome were prepared using differential centrifugation. The microsomal pellets obtained were re-suspended in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.5 mM EDTA and 20% (w/v) glycerol for evaluating activities of hepatic metabolizing enzymes (phase I and II) [7]. Total CYP450 content was estimated in microsomes [14]. Microsomal NADPH Cyt c reductase assay was carried out as
described previously [15]. Aminopyrine demethylase assay in post-mitochondrial supernatant was performed by using colorimetric procedure [16]. Aniline hydroxylase assay in post-mitochondrial supernatant was measured as described previously [17]. Liver microsomal UDP-glucuronosyl transferase (UDP-GT) activity was assessed as described earlier [18]. Glutathione S-transferase (GST) activity was estimated in cytosol [19]. Reduced glutathione (GSH) content was measured in liver homogenate [20].

2.3.2. ALT, AST and LDH

AST and ALT activities in liver homogenate were estimated using colorimetric method [21]. LDH activity was calculated in liver homogenate by the method described previously [22].

2.3.3. Total protein

Total protein content in liver homogenate and hepatic cellular fractions (post-nuclear supernatant, post-mitochondrial supernatant and microsomes) was estimated [23] as described previously.

2.4. Immunoblotting of CYP2C9 and cytokines/chemokines

Western blot analysis was carried out to analyze the time-dependent change in protein expression levels of CYP2C9 and various cytokines/chemokines viz. tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-2 (IL-2) and interferon-γ (IFN-γ) on exposure to CHH. Liver microsomes were prepared at 4 °C by differential centrifugation and total protein content of the microsomes was estimated [23]. The microsomal samples were mixed in sample buffer containing 1% SDS, 2% 2-mercaptoethanol and 10% glycerol and were heat reduced in boiling water bath. The gel and running buffer contained 0.1% and 0.2% SDS, respectively. The standard protein markers (Broad range 200–6.9 kDa, BIO-RAD, Hercules, CA, USA) were used to determine molecular weight.

The sample proteins were electrophoretically resolved on 4% (v/v) stacking and 8% (v/v) separation polyacrylamide gels and electro-transferred onto a nitrocellulose membrane (Protran, Whatman, Germany) pre-soaked in transfer buffer (20% methanol, 0.3% Tris and 1.44% glycine), using semi-dry transblot module (BIO-RAD, Hercules, CA, USA). Then the membranes were blocked with 5% non-fat milk, washed with phosphate buffered saline Tween (PBS-T) (0.01 M phosphate buffer saline, pH 7.4 and 0.01% Tween-20) and probed with primary rabbit polyclonal anti-CYP2C9, anti-TNF-α, anti-IL-1β, and primary goat polyclonal anti-IL-2, anti-IFN-γ antibodies respectively (1:1000 dilution) for 3 h at room temperature. Bound antibodies were detected using respective anti-lgG conjugated with horseradish peroxidase (1:5000 dilution) and immunoreactive bands were developed by chemiluminescent peroxidase substrate kit (Sigma–Aldrich, St. Louis, MO, USA) [7] and exposed to X-ray films (Kodak, Rochester, NY, USA). Quantification was performed by densitometric analysis using ImageJ software program (NIH, Bethesda, MD, USA).

2.5. Histopathological evaluation

The animals of all normoxic and hypoxic groups (ten animals/group) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused intracardially with 0.1 M PBS (pH 7.4) followed by 4% formaldehyde. The liver was carefully dissected out and post-fixed in 4% formaldehyde for 48 h, processed and then embedded in paraffin. Serial tissue sections of 5-μm thickness were prepared and stained with haematoxylin and eosin (H&E). Liver sections were observed for histological changes under light microscope (Axioskop, Carl Zeiss Microscope, Thornwood, NY, USA) and photomicrographs were taken.

2.6. Statistical analysis

The pharmacokinetic analysis was conducted using WinNonlin pharmacokinetic software Version 5.1, Scientific Consultants, MD, USA. Data were presented as mean ± SEM. The statistical significance between normoxic and hypoxic groups was performed by Student-t test using Graph Pad Prism 2.01 (Graph Pad Software Inc., La Jolla, CA, USA). A p-value <0.05 was considered statistically significant.

3. Results and discussion

Hypoxia induces a cascade of structural and functional changes in the physiological system of an organism. Hence, HA-induced hypobaric hypoxia has been associated with various pathophysiological conditions. Diminished supply of oxygen under HA-induced hypoxia, affect physiology of body and render liver more vulnerable to stressful oxidative conditions [3]. This further affects liver physiology and influence hepatic rate of drug detoxification as well as alters vulnerability of liver cells to injury. Previously, our observations showed alteration of DMPK under acute hypobaric hypoxia (24 h) exposure [7]. Other studies have also shown that hypoxia plays a crucial role in pharmacological implication of drugs under hypobaric hypoxia [6]. Both acute and chronic hypoxia affects the processes of drug metabolism and the activity of the enzymes involved in their metabolism [5,7]. This may interrupt the drug disposition process, due to which the effective and safe therapeutic dose-regime may also get affected. Studying the impact of CHH on the hepatic DMPK of orally administered drugs by comparing it with normoxic conditions in experimental models would play a significant role in understanding and rationalizing the drug therapy for people ascending to HA. Therefore, the present study was conducted to evaluate the effect of CHH on DMPK of ibuprofen. This study was further extended to evaluate the extent of liver injury, protein expression levels of CYP2C9 (CYP450 isofrom) and the involvement of various cytokines/chemokines viz. IL-1β, IL-2, IFN-γ, TNF-α.

3.1. Body weight and liver/body weight ratio under CHH

As shown in Fig. 1a and b, with the increase in duration of exposure to CHH, the rat body weight decreased about 23% following 7 days of exposure and by 25% following 14 days of hypoxia exposure. Liver/body weight ratio of rat showed insignificant reduction of 11% after 7 days exposure and 17% after 14 days hypoxia exposure.

3.2. PK of ibuprofen under CHH

Ibuprofen, a NSAID is a well-known, extensively used and well tolerated therapeutic agent used to treat pain and inflammatory diseases. It has also been observed to be useful and efficacious for treatment of HA-induced headache [9,10]. In the present study the influence of CHH on PK of ibuprofen has been investigated. To assess the effect of CHH on PK parameters of ibuprofen, plasma samples from 7 and 14 days CHH exposed rats were analyzed by LC–MS/MS method.

Our observations indicated statistically significant alterations in all PK parameters measured in plasma samples, after both 7 and 14 days durations of CHH exposure as compared with the normoxic group. These alterations in PK parameters indicate the possibility of vasodilation being a more responsible factor, as hypoxic vasodilation is a well known adaptive response to the insufficient oxygen availability [24]. Ibuprofen has been reported to have high-protein binding (98–99%) tendency. Highly protein-bound and renally eliminated drugs are reported to be susceptible to physiological
changes experienced by body at HA, due to which drug Cl decreases significantly [25]. Similar observations were also noticed in the present study where ibuprofen Cl from blood plasma decreased significantly by 29% at 7 days and 44% at 14 days (p < 0.05) in hypoxic group (Table 1). In addition, 1.8-fold and 2.3-fold (p < 0.05) increase was observed in Vd after 7 and 14 days CHH exposure, respectively (Table 1). The hypoxic vasodilation as well as lipophilicity could be responsible for the increased Vd observed for ibuprofen in the CHH exposed groups. A similar increase in Vd of Propofol (an anesthetic agent having similar log p value of ibuprofen, X-log p values of Pubchem) compared under hypoxic conditions in experimental model has been reported earlier [24]. The vasodilation induced by hypoxia can also be accompanied by adrenergic stimulation. Severe hypoxia related increase in adrenergic drive causing decreased hepatic blood flow has been reported [24]. Therefore, the decreased regional blood flow to liver might have been responsible for the lowered energy state of liver mitochondria [6]. Further, T½ of ibuprofen increased significantly by 71% and 90% (p < 0.05) after 7 and 14 days of CHH exposure, respectively, as compared to normoxic rats (Table 1). This may have clinical relevance as it is associated with time to reach steady state concentration and is used to establish a proper dosage regimen of a drug. Moreover, T½ depends directly on Vd and inversely on Cl. The observed increase in Vd and decrease in Cl explains elevation in T½ of ibuprofen. It was also observed that MRT increased significantly by 1.7-fold and 2-fold (p < 0.05) after 7 and 14 days of CHH exposure, respectively (Table 1). Clmax (cal) diminished significantly by 55% (p < 0.05) after 14 days hypoxia (Fig. 2a). Tmax (cal) also found to be insignificantly decreased in 7 and 14 days hypoxia exposed groups (Fig. 2b). AUC (observed) 0-t significantly decreased after 14 days of CHH exposure by 69% (p < 0.05) (Fig. 2c). The results of present study are in accordance with the earlier reports conducted under acute and chronic hypoxic conditions of drugs like acetazolamide [8], propofol [24], furosemide [25], mepridine [26] at HA, which reported altered disposition of these drugs. To gain a detailed insight of affected PK under CHH, carboxy-ibuprofen/ibuprofen ratios were analyzed. An increased ratio of carboxy-ibuprofen/ibuprofen was observed after 7 and 14 days CHH exposure (Fig. 2d). This indicates that decrease in phase II metabolism by UDP-GT could be associated with the affected elimination of carboxy-ibuprofen metabolite.

### 3.3. Phase I and II drug metabolizing enzymes under CHH

Metabolic enzymes protect the cells from toxic compounds however hypoxia have been known to alter the activities of the metabolic enzymes. The results of the present study demonstrated that CHH induces a time-dependent decrease in hepatic metabolism of ibuprofen as evident by a significant reduction in activities of both phase I and II enzymes.

Exposure to CHH significantly decreased CYP-mediated metabolism as indicated by decrease in total CYP450 content by 19% and 28% (p < 0.05) in 7 and 14 days hypoxia exposed rats, respectively (Table 2). The prolonged exposure to hypobaric hypoxia has crucial effect on total hepatic CYP450 content due to direct involvement of oxygen in CYP450 mediated drug metabolic pathways. Further, aniline hydroxylase and NADPH Cyt c reductase activities were significantly decreased by 33% and 16% respectively (Table 2) after 14 days of hypoxia exposure. However, no significant change was observed in aniline hydroxylase and NADPH Cyt c reductase activities after 7 days hypoxia (Table 2) and in aminopyrine demethylase (Table 2) after either duration of hypoxia exposure. The small changes in NADPH, Cyt c reductase and aminopyrine demethylase activities after CHH indicate that chronic hypoxia has minor effect on the activities of these enzymes. Whereas, prolonged duration of hypoxia has significant effect on

### Table 1

| Effect of chronic hypobaric hypoxia (7 and 14 days exposure) on pharmacokinetics (PK) of ibuprofen in rats. |
|---|---|---|---|
| PK parameter | Normoxia | Hypoxia (7 days) | Hypoxia (14 days) |
| Half-life (T1/2): (h) | 1.81 ± 0.09 | 2.89 ± 0.3* | 3.35 ± 0.4 |
| Clearance (Cl): (ml/min) | 2.62 ± 0.19 | 2.03 ± 0.05 | 1.83 ± 0.14* |
| Volume of distribution (Vd): (lt) | 0.405 ± 0.03 | 0.88 ± 0.04 | 2.36 ± 0.34* |
| Mean residence time (MRT): (h) | 2.48 ± 0.12 | 3.39 ± 0.24 | 5.17 ± 0.17* |

* Value are mean ± SEM; n = 6 per group.
* p < 0.05 compared with normoxic rats.
the activity of aniline hydroxylase as a consequence of reduced oxygen availability.

Since function of phase II pathways are dependent on availability of high energy co-factors and reducing equivalents (viz. ATP and NAD) synthesized in mitochondria, therefore, CHH effects on mitochondrial oxygenation characteristics could have further influence on the detoxification systems [6]. UDP-GT is a vital enzyme in conjugation pathway of ibuprofen. Hypoxia causes a decrease in UTP and glucose availability for synthesis of UDP-glucose and UDP-glucuronic acid [27]. In the present study microsomal UDP-GT activity was found to be decreased by 22% and 30% (p < 0.05) after 7 and 14 days hypoxia exposure respectively (Table 3). This might be a contributing factor in the hindered metabolism of ibuprofen under CHH conditions. Furthermore, a significant decrease in GST activity by 40% and 48% (p < 0.05) at 7 and 14 days hypoxia exposure respectively in cytosolic fraction of

Table 2
Effect of chronic hypobaric hypoxia (7 and 14 days exposure) on phase-I hepatic metabolism of ibuprofen in rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normoxia (7 days)</th>
<th>Hypoxia (7 days)</th>
<th>Normoxia (14 days)</th>
<th>Hypoxia (14 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total CYP content (nmol mg⁻¹ protein)</td>
<td>0.84 ± 0.01</td>
<td>0.68 ± 0.02</td>
<td>0.83 ± 0.01</td>
<td>0.60 ± 0.01</td>
</tr>
<tr>
<td>NADPH cytochrome c reductase (nmol cytochrome c reduced min⁻¹)</td>
<td>186.07 ± 2.14</td>
<td>179.14 ± 3.07</td>
<td>194.49 ± 3.35</td>
<td>162.61 ± 2.25</td>
</tr>
<tr>
<td>Aniline hydroxylase (nmol min⁻¹ mg⁻¹ protein)</td>
<td>1.85 ± 0.11</td>
<td>1.41 ± 0.2</td>
<td>2.33 ± 0.06</td>
<td>1.57 ± 0.03</td>
</tr>
<tr>
<td>Aminopyrine demethylase (nmol min⁻¹ mg⁻¹ protein)</td>
<td>8.39 ± 0.15</td>
<td>7.67 ± 0.32</td>
<td>11.02 ± 0.3</td>
<td>9.75 ± 0.34</td>
</tr>
</tbody>
</table>

Value are mean ± SEM; n = 10 per group.

* p < 0.05 compared with normoxic rats.
Table 3
Effect of chronic hypobaric hypoxia (7 and 14 days exposure) on phase-II hepatic metabolism of ibuprofen in rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normoxia (7 days)</th>
<th>Hypoxia (7 days)</th>
<th>Normoxia (14 days)</th>
<th>Hypoxia (14 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDP-glucuronosyl transferase (UDPGT) (nmol min⁻¹ mg⁻¹ protein)</td>
<td>0.49 ± 0.03</td>
<td>0.38 ± 0.01</td>
<td>0.48 ± 0.01</td>
<td>0.33 ± 0.01</td>
</tr>
<tr>
<td>Glutathione S-transferase (GST) (Units mg⁻¹ protein)</td>
<td>26.57 ± 0.52</td>
<td>15.89 ± 0.49</td>
<td>28.26 ± 0.9</td>
<td>14.83 ± 0.2</td>
</tr>
<tr>
<td>Reduced glutathione (GSH) (µg gm⁻¹ protein)</td>
<td>4.13 ± 0.12</td>
<td>3.22 ± 0.23</td>
<td>5.14 ± 0.17</td>
<td>3.59 ± 0.27</td>
</tr>
</tbody>
</table>

Value are mean ± SEM; n = 10 per group.
* p < 0.05 compared with normoxic rats.

Table 4
Effect of chronic hypobaric hypoxia (7 and 14 days exposure) on aspartate transferases (AST), alanine transaminases (ALT), lactate dehydrogenase (LDH) in rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normoxia (7 days)</th>
<th>Hypoxia (7 days)</th>
<th>Normoxia (14 days)</th>
<th>Hypoxia (14 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartate transferases (AST) (IU/L)</td>
<td>8.75 ± 0.13</td>
<td>12.49 ± 0.05</td>
<td>9.06 ± 0.16</td>
<td>13.41 ± 0.12</td>
</tr>
<tr>
<td>Alanine transaminases (ALT) (IU/L)</td>
<td>9.46 ± 0.06</td>
<td>10.51 ± 0.06</td>
<td>10.03 ± 0.06</td>
<td>11.86 ± 0.17</td>
</tr>
<tr>
<td>Lactate dehydrogenase (LDH) (nmol/mg protein)</td>
<td>44.34 ± 1.43</td>
<td>59.27 ± 1.89</td>
<td>42.19 ± 1.09</td>
<td>58.65 ± 2.02</td>
</tr>
</tbody>
</table>

Value are mean ± SEM; n = 10 per group.
* p < 0.05 compared with normoxic rats.

Rat liver was also observed (Table 3). This signifies the crucial role played by essential co-factors, such as NAD and ATP in glutathione conjugation. Impaired mitochondrial function under hypoxic conditions results in decrease in ATP supply [3]. The decrease in GST activity might be due to depleted level of GSH, which serves as a coenzyme for GST to dispose-off different drug metabolites. Similarly, following 7 days of CHH exposure, 22% (p < 0.05) reduction was obtained in the reduced GSH concentration in rats followed by further decrease of 30% (p < 0.05) after 14 days of hypoxia exposure (Table 3) as an effect of hypoxia per se.

Fig. 3. (a) Effect of chronic hypobaric hypoxia exposure (7 and 14 days) on CYP2C9 protein expression level as determined by Western blotting. Value are mean ± SEM; n = 10 per group. * p < 0.05 compared with normoxic rats. (b) Effect of chronic hypobaric hypoxia exposure (7 and 14 days) on IFN-γ, TNF-α, IL-2 and IL-1β expression level as determined by Western blotting. Corresponding densitometric graph as analyzed by ImageJ software. Value are mean ± SEM; n = 10 per group. * p < 0.05 compared with normoxic rats.
3.4. ALT, AST and LDH under CHH

For investigation of lysosomal destruction and cellular deterioration, ALT and AST levels were studied. Liver tissue obtained from hypoxic and normoxic rats were assessed for hepatic injury through analysis of ALT, AST and LDH levels. ALT levels were significantly increased by 42% and 48% (p < 0.05) at 7 and 14 days CHH exposure (Table 4). An increase in ALT levels by 11% and 18% (p < 0.05) was observed after 7 and 14 days hypoxia exposure, respectively (Table 4). LDH activity was also found to be increased by 33% and 39% (p < 0.05) after 7 and 14 days exposure to CHH (Table 4). Elevated levels of these enzymes under hypoxic condition proved that hypoxia causes disruption of lysosomes concurrently with changes in cell membrane permeability and release of these enzymes. Increased LDH activity was also observed after CHH exposure indicating LDH leakage, which signifies the effect of hypoxia per se.

3.5. CYP2C9 and cytokine/chemokine expression levels under CHH

CYP2C9 plays an important role in metabolism of wide variety of therapeutic drugs including NSAIDs. It is mainly responsible (>70%) for the oxidative metabolism of ibuprofen [28]. It forms the two metabolites of ibuprofen, 3- and 2-hydroxyibuprofen. The 3-hydroxy metabolite once formed is metabolized almost completely to the corresponding carboxy derivative via cytosolic dehydrogenases [28]. The observations of present study indicate that CHH impairs CYP-mediated hepatic metabolism of ibuprofen but did not reveal complete information on the cause underlying these alterations. Therefore, further investigation has been performed to elucidate the underlying mechanism associated with protein expression of specific CYP450 isofrom (i.e., CYP2C9) related to ibuprofen metabolism. A significant decrease in total CYP450 content was observed, hence, further study was conducted to analyze the expression of CYP2C9 protein levels in CHH exposed groups as compared with normoxic groups.

In our study, Western blot analysis revealed a significant downregulated expression of CYP2C9 protein by 23% (p < 0.05) after 7 days of CHH exposure (Fig. 3a). CHH exposure of 14 days further attenuated the expression of CYP2C9 by 39% (p < 0.05) (Fig. 3a). This implies that CHH affect CYP2C9-mediated ibuprofen metabolism. These results are in agreement with earlier studies which have demonstrated that activity and expression of isofroms of CYP450 get affected under hypoxia [7,11]. The role of hypoxia in altering CYP450-mediated metabolism of drugs has been shown and it has implicated the fact that the modulation of CYP450 activity and expression is possibly multi-factorial. This reduction in catalytic activity of different isofroms of CYP450 occurs by dual mechanism, an early reduction in activity and a later decrease in their expression, both triggered by cytokines viz. IFN-γ, IL-1β and IL-2 which activate signaling of PTK, PKC and MAPK and the production of reactive oxygen intermediates (ROI) [11]. HA-induced hypobaric hypoxia has been accounted to be an important factor in DM, as hypoxia prompts the release of numerous cytokines, such as IL-1β, IL-2, IL-4, IL-5, IL-6, IFN-γ, TNF-α and erythropoietin (Epo) [29]. A significant enhanced expression level of inflammatory cytokines/chemokines viz. IFN-γ, TNF-α, IL-2 and IL-1β by 1.6, 1.5,
and alters hypoxia oxygen Illustration interleukin-2 observations conditions. 3.6. Hepatic histopathological observations under CHH

Hepatic damage is another physiological effect encountered under hypoxic conditions. Under lower O₂ concentration gradient, the liver is perfused with partially deoxygenated portal blood, which makes it prone to damage caused due to chronic hypoxic conditions. Moreover, glucuronidation pathway is localized to hepatic centrilobular regions and during chronic hypoxia, reduced O₂ supply decreases glucuronidating activity which could further exacerbate centrilobular necrosis [6].

The integrity of liver tissue under hypoxic stress was identified by histopathological study of the liver samples of rats exposed to different durations of CHH exposure. The present histological observations showed a time-dependent alteration in histopathology of liver under CHH. Our study revealed that the liver sample from 7 days (Fig. 4a) and 14 days (Fig. 4b) normoxia animal exhibited normal liver cell architecture with no signs of necrosis, fatty acid change, haemorrhage or vascular congestion etc. However, slight sinusoidal dilatation was observed in animals treated for 14 days with ibuprofen under normoxic conditions. After exposure to CHH, the general architecture of liver tissue samples was intact and normal. However, histopathological damage including mild necrosis of hepatocytes, slight haemorrhage and moderate degree of inflammation around central vein was observed in the liver tissues of rats exposed to 7 days (Fig. 4c) CHH. On the other hand, profound hepatic damages were observed after 14 days CHH (Fig. 4d). The liver samples demonstrated damage including evident sinusoidal dilatation, vascular congestion and presence of apoptotic hepatocytes. However, no fatty change of centrilobular region could be observed in any liver samples. These results are similar to findings of earlier reports of liver damage including lobular inflammation, fibrosis, swelling of hepatocytes and necrosis etc. under chronic intermittent hypoxia which explained that hypoxia plays an important role in the drug metabolizing mechanism [30].

4. Conclusion

Although the altered DMPK of drugs under hypoxic conditions is being identified, the physiological functions and signaling
pathways that mediate such responses remain largely unknown. Additional experiments are desirable to further evaluate these observations. In summary, while the precise basis for such alterations in the ability of a person to metabolize drugs under stressful conditions has not been elucidated, our study provides the evidence that PK and hepatic DM of ibuprofen gets affected under HA-induced CHH. Moreover, our data shows elevated liver ALT, AST and LDH levels as well as undesirable histopathological effects in liver after prolonged exposure to hypobaric hypoxia. The results also suggest impairment of phase I metabolism associated with CYP2C9 due to down-regulation of this protein. The enhanced protein expression of cytokines/chemokines viz. IFN-γ, TNF-α, IL-1β and IL-2 could be one of the factors responsible for observed down-regulation of CYP2C9 expression under CHH conditions. The precise molecular mechanism of CYP-mediated attenuation in CHH, however, needs to be further delineated. Marked alterations in DMPK of ibuprofen imply that the safe and effective therapy under CHH could be hindered. The information obtained about the attenuation of these mechanisms could be crucial for the use of optimal and safe dosage of therapeutic drugs under adverse environmental stresses such as HA-induced hypobaric hypoxia (collectively depicted in Fig. 5). Further, the human studies to evaluate their susceptibility toward hypobaric hypoxia would be helpful to explore safe and effective therapy of commonly used drugs at HA.

Conflict of interest

The authors declare that there are no conflicts of interest.

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