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Transcriptional downregulation of sterol metabolism genes in murine liver exposed to acute hypobaric hypoxia

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Abstract

Ascent to high-altitude results in decreased inspired partial pressure of oxygen because of a decrease in barometric pressure. Altitude acclimatization requires physiological and metabolic changes to improve tolerance to altitude hypoxia. Cellular response to hypoxia results into changes in the profile of gene expression and the present study explored the same in murine model. Liver being the largest metabolic organ, the molecular details of acute hypobaric hypoxia (AHH) induced transcriptional changes in the tissue were investigated. Swiss albino mice were exposed to hypobaric hypoxia (~426 mmHg) in a decompression chamber and cDNA microarray was used to study the transcriptional profile in liver. Notably, by the tenth hour several of the genes involved in sterol metabolism such as SREBF1, INSIG1, HMGCS1, FDFT1, SQLE, and HSD3B4 were downregulated more than 2-fold suggesting that AHH suppresses sterol biosynthesis in the liver. Real-time PCR helped validate the downregulation of SREBF1, HMGCS1, FDFT1, and HSD3B4 genes. However, no significant change was observed in the serum cholesterol levels throughout the AHH exposure. The findings are indicative of transcriptional downregulation of SREBP target genes as a part of acclimatization response to hypoxia. The study highlights the significance of SREBP in the regulation of sterol metabolism under the acute hypoxic response.

Keywords: High-altitude; Acute hypobaric hypoxia; Sterol; SREBP

High-altitude (HA) environment is characterized by hypobaric hypoxia. The low barometric pressure at HA brings about reduction in the partial pressure of oxygen in inspired air [1]. Hence, exposure to extreme altitude causes severe hypoxemia, respiratory alkalosis, and greatly reduced maximal oxygen consumption [2]. Acclimatization to hypoxia is of considerable clinical relevance, as it influences the pathophysiology of anaemia, polycythaemia, tissue ischaemia [3,4]. Un-acclimatized sojourners may develop cerebral and pulmonary syndromes shortly after ascent to HA [5]. Hypoxia stresses the metabolic processes of an organism for want of oxygen and elicits a wide range of adaptive responses at the systemic, tissue, and cellular level [6]. A number of transcription factors including hypoxia inducible factor 1 (HIF1), activating protein 1 (AP1), nuclear factor-κB (NF-κB), early growth response 1 (EGR1) are also modulated by changes in pO_2, either directly or indirectly [7]. HIF1 functions as a global regulator of O_2 homeostasis facilitating both O_2 delivery and adaptation to O_2 deprivation [8]. Genome wide expression analysis has revealed significant information regarding changes in global transcriptome profile in response to hypoxia, however, many uncertainties regarding the changes that occur in peripheral tissues in mammals in response to acute hypobaric hypoxia (AHH) are yet to be elucidated.

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Hypoxia responses are thought to be evolutionarily conserved in all mammalian cells. However, different tissues of the organism are exposed to different ranges of oxygen concentration and have varied energy requirements [9]. Liver, the largest metabolic organ in the body performs a number of important and complex biological functions that are essential for survival. It has an important role in metabolism of carbohydrates, proteins, lipids, drugs, etc. Previous studies have analyzed the effect of hypoxia on different hepatocellular carcinoma cell lines [10–12]. However, no *in vivo* studies have been conducted so far to find the effect of AHH on hepatocyte gene expression. In the present study, we employed cDNA microarrays to investigate the variations in the transcriptome profile in murine liver in response to 10 h of AHH, equivalent to ~4600 m (i.e., 426 mmHg). Though the array results revealed the differential expression of a number of genes involved in diverse metabolic pathways, the downregulation of sterol regulatory element binding protein (SREBP) target genes appeared distinct as a part of acclimatization response to AHH. The transcription profiles of these genes have been explored at the fifth and tenth hour of AHH exposure by real-time PCR. Additionally, serum cholesterol levels were measured.

Materials and methods

Exposure of mice to hypobaric hypoxia and RNA isolation. Eight weeks old male albino Swiss mice ($n = 5$ for each group) were exposed to simulated altitude of 15,000 ft (~4600 m). The pressure in the decompression chamber was maintained at 426 mmHg for a period of 5 and 10 h. Controls as well as the experimental animals were kept on fasting. At the end of each exposure the mice were sacrificed by cervical dislocation. Blood was collected and after separating the serum the samples were kept at −70 °C until analysis. Total RNA from both the groups was isolated immediately from dissected livers using TRIzol reagent (Invitrogen, USA). The Institute's Animal Ethics Committee approved the animal procedures.

Microarray experiments. Equal amount of total RNA from the five mice of 10 h experimental and control group was pooled separately to give a total of 6 μg prior to labeling with the Micromax NEN TSA labeling kit (Perkin-Elmer Life Sciences, USA). The labeled cDNA probes were mixed and simultaneously hybridized to 15K mouse cDNA arrays (The Microarray Center, Clinical Genomics Center, University Health Network, Toronto, Canada) in an overnight incubation. The microarrays were scanned using GenePix Professional 4200A scanner (Axon Instruments, USA). Microarray image acquisition and analysis was performed using GenePix Pro 6.0 software (Axon Instruments, USA). GenePix result files (.gpr) were then transferred to TIGR Express Converter to generate TIGR MultiExperiment Viewer file (.mev) as output. Data normalization (lowess), flagging, filtering, and flip-dye consistency check were performed using TIGR MIDAS [13]. Each slide carried at least two spots for each gene. In-slide replicates analysis which is a replica averaging function that works on data files with replicated spots was used to group and merge the intensities of the replicated spots. Then flip-dye consistency checking function was performed to filter out spots that showed expression level inconsistency between a pair of flip-dye replicates. Normalized and filtered expression files were analyzed using TIGR MeV [13]. Genes showing consistency between dye swap experiments and upregulated or downregulated at and more than 2-fold were selected. We used Onto-Express [14,15] to functionally classify the differentially expressed genes according to the gene-ontology (GO) categories: biological process, cellular role, and molecular function. GenMAPP software was used to construct a pathway (MAPP) depicting sterol synthesis and to view the gene expression data on the MAPP [16].

Real-time PCR. Pooled RNA was prepared as in previous experiment for the fifth hour, tenth hour, and the control mice. Single strand cDNA was reverse transcribed using First-Strand cDNA Synthesis kit (Amersham Biosciences, UK). Primers for real-time PCR were designed for SREBF1, HMGCS1, FDFT1, and HSD3B4 genes using the Primer Express™ software (Perkin-Elmer Applied Biosystems, USA) and are listed in Table 1. Real-time PCRs were performed using SYBR Green PCR Master Mix (Applied Biosystems, USA) on ABI Prism 7300 Sequence Detection System (Applied Biosystems, USA). For each 25 μl PCR, 2.0 μl cDNA, 1.0 μl sense and antisense primer (2.5 μM each), 12.5 μl SYBR Green PCR Master Mix, and 9.5 μl PCR-grade water were used. The cycling conditions were: 94 °C for 10 min, followed by 40 cycles of 94 °C for 15 s, 60 °C for 30 s. The PCRs were performed in triplicate for each gene. Relative transcript quantities were calculated using the $\Delta\Delta C_t$ method with β-actin (ACTB) as the endogenous reference gene amplified from the samples.

Cholesterol estimation. Enzymatic determination of serum cholesterol was performed using a commercial kit (Bayer Diagnostics, India).

Statistical analysis. The data from real-time PCR and cholesterol estimation are expressed as means ± SD and evaluated using Student's t test. The level of significance was set at $p < 0.05$.

Results and discussion

Differential gene expression analysis of murine liver exposed to AHH

Acute hypobaric exposure of 10 h had a major impact on the liver because a number of genes involved in various biological processes such as metabolism, signal transduc-

Table 1
Primers used for real-time PCR of sterol metabolism genes

Gene	Primer sequence	Product size (bp)
SREBF1	F: 5′-TTCAGTCCAGGTAGCTCACGGT-3′	146
	R: 5′-TGGCCTCATGTAGGAATACCCT-3′	
HMGCS1	F: 5′-CCCCTTCACAAATGACCACAG-3′	136
	R: 5′-GACAGCTGATTCAGATTCGGC-3′	
FDFT1	F: 5′-GCCTGCCGTCAAAGCTATCATA-3′	104
	R: 5′-TCTTGGAGATGACCTGCTTGGT-3′	
HSD3B4	F: 5′-ATCCACACCGCTGCTGCTATT-3′	125
	R: 5′-ATGAATGTTGGCACGTTGGC-3′	
ACTB	F: 5′-TTGCTGACAGGATGCAGAAGG-3′	149
	R: 5′-GCTGATCCACATCTGCTGGAA-3′	

F, forward primer; R, reverse primer. Primers were designed using Primer Express software using sequence data from the Ensembl database.

tion, transcription, apoptosis, cell cycle, and ubiquitination were found to be differentially expressed (Supplementary Table S1). Out of the 15,000 genes analyzed, 512 genes (~3.5%) were induced or repressed 2-fold or more. Using 2-fold change as the cut-off value, several genes involved in sterol metabolism (Table 2) were found to be downregulated, whereas, transcript levels of genes involved in fatty acid biosynthesis were unaffected (Supplementary Table S1). The major sterol metabolism genes identified to be downregulated by microarray analysis are acetoacetyl-CoA thiolase (ACAT2), 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1 (HMGCS1), farnesyl diphosphate farnesyl transferase 1 (FDFT1), squalene epoxidase (SQLE), sterol-C4-methyl oxidase-like (SC4MOL), and 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (HSD3B4) with fold change of −2.88, −4.44, −2.50, −2.18, −3.16, and −27.62, respectively. Sterol regulatory element binding factor 1 (SREBF1), also known as SREBP1 and insulin induced gene 1 (INSIG1) which play crucial role in lipid homeostasis were also downregulated by more than 3-fold.

The transcription profiles of SREBF1, HMGCS1, FDFT1, and HSD3B4 were explored at the fifth and tenth hour of AHH exposure by real-time PCR. A comparison of the $\Delta\Delta C_t$ values of these four genes (Fig. 2A) at the tenth hour of exposure to AHH (fold change: −4.11, −2.3, −4.53, and −83.19, respectively) with the fifth hour (fold change: −1.24, −1.13, −1.85, and −1.39, respectively) revealed a significant downregulation (p value $\leqslant 0.01$). The trend of expression of the genes was same as those obtained by microarray analysis for the tenth hour indicating that the results of microarray analysis were consistent, although, the amplitude of change differed between the two methods (Fig. 2B).

Pathway analysis of genes involved in sterol metabolism

Based on the present findings and the available literature the pathway for sterol biosynthesis was generated using GenMAPP software and is depicted in Fig. 1. The genes identified by microarray analysis can be seen playing a major role in cholesterol synthesis. The first step involves synthesis of acetoacetyl-CoA by the enzyme ACAT2 [17], and we observed transcriptional downregulation of ACAT2. In the subsequent step, 4.4-fold downregulation of HMGCS1 was observed that regulates the generation of HMG-CoA from acetyl-coA and acetoacetyl-CoA [18]. Further, FDFT1 catalyzes the condensation of farnesyl pyrophosphate, yielding squalene [19], which is converted to 2,3(*S*)-oxidosqualene by the enzyme SQLE [20]. AHH also resulted in decreased mRNA expression of SEC14-like 2 (SEC14L2), a cytosolic protein which stimulates SQLE [21]. These steps ultimately lead to cholesterol synthesis. Further, in the biosynthesis of hormonal steroids, HSD3B4, a 3-ketosteroid reductase rather than a 3β-hydroxysteroid dehydrogenase/isomerase, catalyzes the conversion of dihydrotestosterone, an active androgen to 5α-androstanediol in the presence of the cofactor NADPH [22] and this gene was also repressed under the AHH (Fig. 1).

Another important observation was the repression of SREBP1, belonging to a family of membrane-bound transcription factors, which regulate lipid homeostasis and of INSIG1 as depicted in Fig. 1. Brown, Goldstein, and their colleagues highlighted the importance of SREBP in the regulation of cholesterol synthesis in their reports [23–27]. In times of sterol depletion, SREBP cleavage-activating protein (SCAP) binds to the N-terminal domain of SREBP in a tight complex. SCAP chaperones SREBP in this complex to the Golgi apparatus, where the latter is cleaved into its mature form by two site-specific proteases namely, Membrane-bound transcription factor peptidase, site 1 (S1P) and membrane-bound transcription factor peptidase, site 2 (S2P) [23]. During this process, INSIG1 (−5.86-fold), an endoplasmic reticulum (ER) protein, binds the sterol-sensing domain of SCAP to facilitate the sterol-dependent retention of the SCAP/SREBP complex in the ER [24]. Moreover, SREBP1 is a potent activator of all SREBP-responsive genes, including those that mediate the synthesis of cholesterol, fatty acids, and triglycerides, whereas SREBP2 preferentially activates cholesterol synthesis [25,26]. It has been shown that the targeted disruption of SREBP1 in mice leads to elevated levels of SREBP2 and hepatic cholesterol while the disruption of SREBP2 gene leads to 100% lethality showing the high potency of SREBP2 [27]. HMGCS1, FDFT1, and SREBP1 have been previously characterized as directly regulated by SREBPs

Table 2
Differentially expressed genes of sterol metabolism in response to 10 h of AHH

Accession No.	Gene symbol	Gene description	LocusLink ID	Fold change
BG073378	ACAT2	Acetoacetyl-CoA thiolase	110460	−2.88
BG069739	HMGCS1	3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 1	208715	−4.44
BG069211	FDFT1	Farnesyl diphosphate farnesyl transferase 1	14137	−2.50
BG064535	SQLE	Squalene epoxidase	20775	−2.18
BG065234	SEC14L2	SEC14-like 2 (*S. cerevisiae*)	67815	−3.48
BG071087	SC4MOL	Sterol-C4-methyl oxidase-like	66234	−3.16
BG071713	SREBF1	Sterol regulatory element binding factor 1	20787	−3.76
BG067488	INSIG1	Insulin induced gene 1	231070	−5.86
BG072680	HSD3B4	3β-Hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase	15495	−27.62

The genes selected are the ones that showed consistent and $\leqslant$−2-fold changes in expression in dye swap experiments.

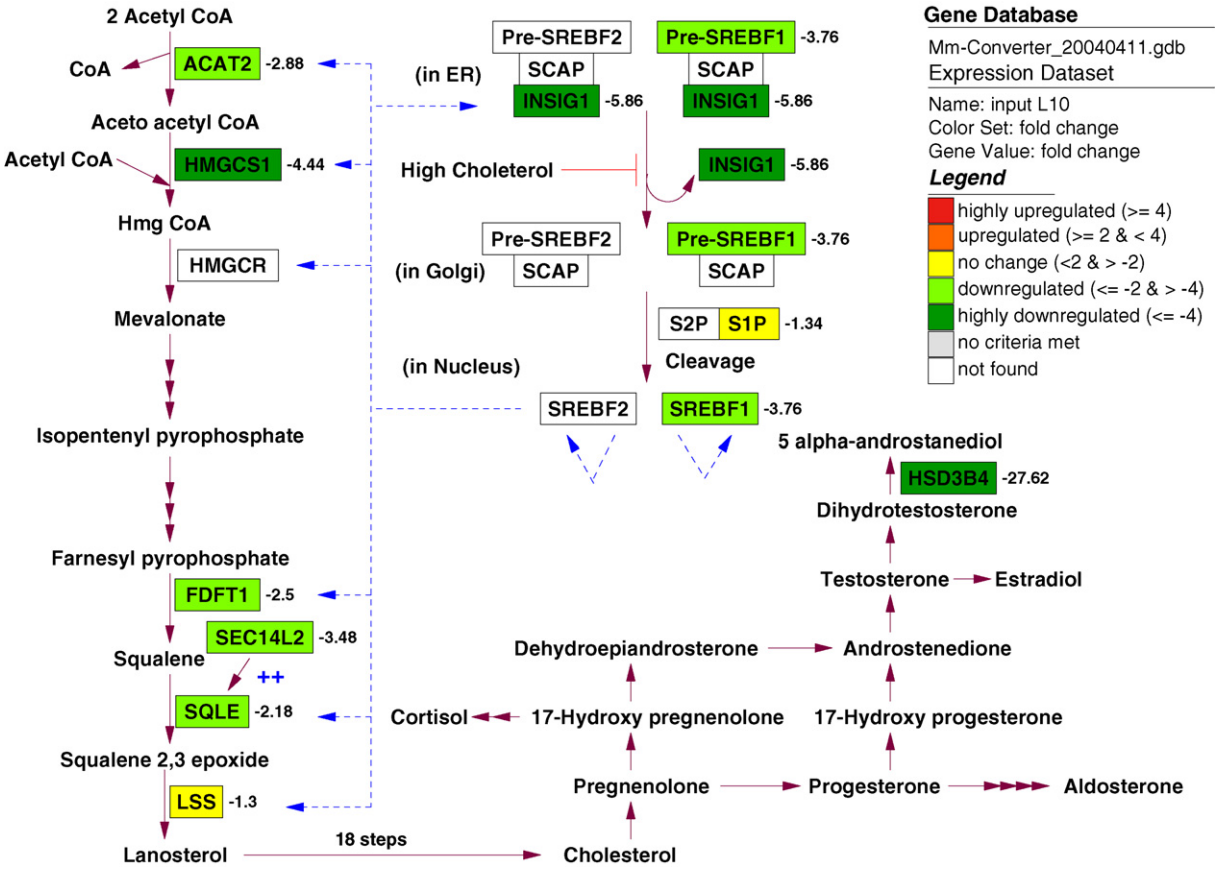

Fig. 1. Schematic diagram of sterol metabolism created by GenMAPP software. The fold change in expression of genes involved in sterol pathway, identified by microarray analysis, in murine liver exposed to 10 h of AHH has been indicated. ACAT2, acetoacetyl-CoA thiolase; HMGCS1, 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1; HMGCR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; FDFT1, farnesyl diphosphate farnesyl transferase 1; SQLE, squalene epoxidase; SEC14L2, SEC14-like 2 (*Sacchromyces cerevisiae*); LSS, lanosterol synthase; HSD3B4, 3β-hydroxysteroid dehydrogenase/ Δ^5-Δ^4-isomerase; SREBF1, sterol regulatory element binding factor 1; SREBF2, sterol regulatory element binding factor 2; INSIG1, insulin induced gene 1; SCAP, SREBP cleavage activating protein; S1P, membrane-bound transcription factor peptidase, site 1; S2P, membrane-bound transcription factor peptidase, site 2 (—|, inhibition; ++, activation; - - -▶, transcriptional regulation).

[28]. The microarray based study from transgenic/knockout mice (transgenic for SREBP1a, transgenic for SREBP2, and knockout for SCAP) has identified INSIG1 and SC4MOL in addition to several other genes as the potential targets of SREBP [29].

Effect of AHH on sterol metabolism

Sterols are vital to cellular membrane physiology, dietary nutrient absorption, reproductive biology, stress responses, salt, and water balance, and calcium metabolism [30]. Cholesterol synthesis, although, requires only one oxygenation step but several such steps are required in its conversion to steroid hormones and subsequent degradation to bile acids [31]. It is, therefore, expected that genes regulating the oxygenation steps in the sterol biosynthesis will be affected under the low oxygen concentrations. It is reported in mammalian cells that hypoxia disturbs cholesterol metabolism in cells and induce intracellular cholesterol accumulation with suppression of its *de novo* synthesis [32]. There are reports of reduction in plasma cholesterol levels after 3 days in human subjects exposed to hypobaric hypoxia [33]. Although the change observed in the serum cholesterol levels at the fifth and the tenth hour was not significant (Fig. 3), we observed a decrease in the transcript levels of SREBP1 and the SREBP target genes as compared to normoxic controls by the tenth hour of AHH exposure. This suggested that AHH affected inactivation of SREBP mediated transcription could be the cause of suppressed *de novo* synthesis of cholesterol which may take time to get reflected in the serum.

According to the experimental evidences provided by Hughes et al. in the fission yeast (*Schizosaccharomyces pombe*), the sterol regulatory element binding protein (SRE1), which is the yeast homolog of mammalian SREBP, mediates adaptation to hypoxia [34]. The model proposed in the study is indicative of activation of SRE1 and its target genes in response to depleted sterol levels or low oxygen in the initial period of adaptation with subsequent increase in the sterol levels. Though mammalian cells and yeast induce hypoxic gene transcription at

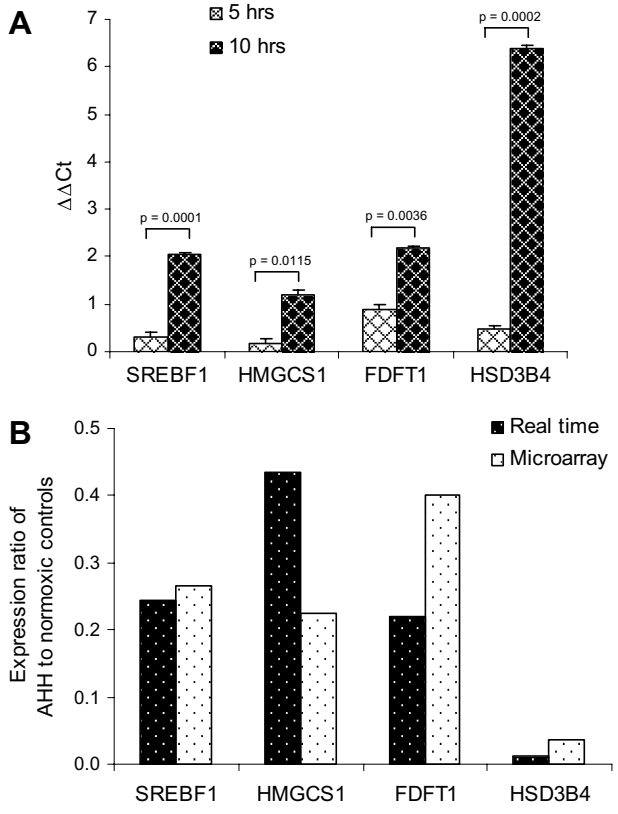

Fig. 2. (A) Comparison of $\Delta\Delta C_t$ values of SREBF1, HMGCS1, FDFT1, and HSD3B4 in mice livers exposed to 5 and 10 h of AHH. Real-time PCR with gene specific primers was performed to determine ΔC_t values in AHH and control mice livers using β-actin as an internal control. Relative mRNA quantities were calculated by the $2^{-\Delta\Delta C_t}$ method. (B) Comparison of ratio of expression of AHH versus control mice liver obtained from real-time PCR and microarray experiments.

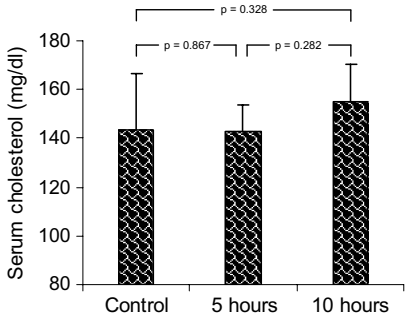

Fig. 3. Serum cholesterol levels (mg/dl) of control, 5 and 10 h AHH exposed mice.

different oxygen concentrations, similar mechanism could be functional in mammals as well.

The transcriptional downregulation of the SREBP target genes by the tenth hour is suggestive of the fact that hypoxia suppresses sterol biosynthesis in the liver by the tenth hour which might lead to the transcriptional and post-transcriptional activation of SREBP and its target genes leading to elevated cholesterol levels at a later time point as a part of adaptation to hypoxia.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.12.159.

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