American Journal of Biochemistry and Biotechnology

Original Research Paper

Role of the Wnt/β-Catenin Signaling Pathway in Reducing Lipogenesis of Obese Rats under Hypoxic Exercise

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Article history
Received: 02-11-2021
Revised: 24-02-2022
Accepted: 25-02-2022

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Abstract: Hypoxic exercise exerts a significant effect on body weight loss and body fat reduction. Methods: In the present study, we constructed an obese rat model and divided rats into the following groups: Normoxic quiet (C), Hypoxic quiet (H), normoxic Exercise (CE), and Hypoxic Exercise (HE). We employed isobaric Tags for Relative and Absolute Quantification (iTRAQ) using proteomic technology to screen the differentially expressed proteins in the four groups. Additionally, we used Reduced-Representation Bisulfite Sequencing (RRBS) to analyze high-throughput DNA methylation profiles in both HE and CE groups. Finally, we screened and analyzed the CpG islands and Differentially Methylated Regions (DMRs) of genes related to the Wnt/β-catenin-signaling pathway. Results: The mRNA levels of gene products related to the Wnt/β-catenin-signaling pathway (i.e., c-myc and β-catenin) were significantly augmented (P<0.05) in the HE group compared to the CE group; the levels of APC and GSK3β were significantly diminished (P<0.05). The expression levels of the Wnt/β-catenin-signaling pathway proteins c-myc and β-catenin were also significantly increased in the HE group relative to the CE group (P<0.05). Among genes related to the Wnt-signaling pathway, the CpG-promoter methylation sites included Fzd2, Fzd9, Wnt16, Wnt2b, Sfrp2, Sfrp1, Sox2, and Sox1; the DMRs included CDC, Fzd10, Gsk-3β, PI3k, Wnt16 and Wnt6. Conclusions: Our present findings provide evidence that hypoxic exercise may inhibit adipogenesis and adipogenesis through canonical Wnt signaling.

Keywords: Hypoxic Exercise, iTRAQ, RRBS, Wnt/β-Catenin Signaling Pathway, Lipid Metabolism

Introduction

The percentage of individuals who are overweight or obese continues to rise globally (Nangunoori *et al*., 2016) and obesity and its complications seriously threaten human health (Upadhyay *et al*., 2018). Studies have shown that plateau and hypoxic exercise are more effective than normal-oxygen exercise in reducing body weight and body fat (Lee *et al*., 2004, Netzer *et al*., 2008). For example, training at an altitude of over 1,700 meters (m) has been shown to reduce body weight by 1.5-3.3% (Lippl *et al*., 2010). As such, hypoxic exercise improves bodily function, reduces cardiovascular and metabolic risk factors, improves lipid metabolism (including lowering blood lipid levels), inhibits fatty acid synthesis, and promotes fatty acid decomposition/oxidation (Haufe *et al*., 2008, Wiesner *et al*., 2010). Possible mechanisms governing weight loss during hypoxic exercise include decreased appetite; increased leptin expression; increased expression of Hypoxia-Inducible Factor (HIF) and Peroxisome Proliferator-Activated Receptor (PPAR), both of which regulate fat metabolism; reduced-fat synthesis; and enhanced fat metabolism (Debevec *et al*., 2014, Gracey *et al*., 2011; Jiang *et al*., 2015; Horscroft and Murray, 2014). Hypoxic environments and hypoxic exercise can inhibit the formation of fat cells, reduce the expression of Fatty Acid Synthase (FAS) mRNA and reduce the synthesis of fat; additionally, hypoxic exercise increases the energy supply available for fatty acid ß-oxidation (Korkushko *et al*., 2010).

The canonical Wnt/β-catenin pathway is a negative regulator of adipocyte differentiation in the liver (Ross *et al*., 2000). Most studies on the effects of hypoxic training on the fat formation and lipid metabolism focus only on a specific protein or signaling pathway and a systematic and comprehensive evaluation of the mechanisms subserving hypoxic weight loss is lacking. In the present study, we

used isobaric Tags in Relative and Absolute Quantification (iTRAQ) proteomics and assessed DNA methylation to investigate alterations in proteins and signaling pathways involved in adipocyte differentiation and lipid metabolism under hypoxic exercise in rats.

Methods

Animal Model and Tissues

Ninety 5-week-old male SD rats were purchased from Wuhan Maihalic Biotechnology Co., Ltd. (http://www.hlkbio.cn/). Rats were housed in separate cages (n = 5). After allowing the rats to acclimatize to their environment for one week (22.0 24.0℃, the lighting time period is 8:00-20:00) and randomly divided into general food control group (n = 10) and nutritional obesity model construction group (n = 80). The general food control group was fed standard animal feed (fat content, 10% of total caloric energy; purchased from Synergy Pharmaceutical Bioengineering Co., Ltd., batch number 20190622), with access to ultrapure drinking water ad lib; while the nutritionally obese rat model group was fed high-fat feed (fat content, 45% of total caloric energy; purchased from Hua Fukang, batch number D12492), with freely available ultrapure drinking water. Then, a standard screening of 50 obese rats was executed and 40 rats were randomly divided into normoxia quiet group (C group), hypoxic quiet group (H group), normoxia exercise group (CE group), Hypoxic Exercise group (HE group), (10 rats per group). If the Lee index, fat mass, and body fat ratio of obese rats were significantly increased ($P<0.05$), it indicated that the above-mentioned obese rat model was successfully established (Lu *et al*., 2014).

Exercise Program

Obese rats in the CE and HE groups exercised on a treadmill for two weeks to adapt to the training regimen, during which, the training speed was increased from 16 m/min to 25 m/min and the training time was gradually extended from 20 min/d to 60 min/d-with 5 min added every 2 d. The exercise group used a horizontal treadmill for endurance training. The training intensity of the CE group was 25 m/min under normoxia, whereas that of the HE group was 20 m/min (the oxygen concentration was 13.6%, equivalent to 3500 m above sea level); and training ensued for 1 h/d, 5 d/wk for four weeks.

Sample Collection

After fasting overnight, all rats were anesthetized by intraperitoneal anesthesia (10% chloral hydrate, 350 mg/kg body weight). The rats were euthanized in a glass jar containing a cotton wool pad soaked in 30% anesthetic ether. Subsequently, both the body length and body weight of each rat were measured, and Lee's index was calculated; then each rat was quickly fixed on a plate with ice cubes, the abdominal cavity was opened, and blood from the abdominal aorta was sampled and serum was collected and stored at 20°C pending further testing. Next, the upper edge of the right liver lobe was removed and quickly rinsed off the blood with refrigerated normal saline. The filter paper was used to absorb the water and samples were then quick-frozen in liquid nitrogen to be stored for follow-up experiments. This study was approved by the Ethics Committee of Shandong Sport University Committee (no. 2015206).

ITRAQ Proteomics

For labeling experiments, the total liver protein was processed with homogenization and SDT lysis buffer (pH = 7.6, 100 mm Tris-HCl,4% SDS) (du Souich and Fradette, 2011). The quality of the extracted protein was determined by SDS-PAGE (Beyotime Biotechnology Co., Ltd., Beijing, China). Then, 30 μg of protein was used from each sample for FASP enzymatic hydrolysis and subsequent peptide quantification (Wisniewski *et al*., 2009). For samples that passed quality control, 100 μg of the peptide was removed from each portion and processed and labeled the samples based on the instructions in the iTRAQ Reagent-4/8 Plex multiplex labeling kit 100 μg of peptide per fraction was extracted, processed, and labeled according to the instructions of the iTRAQ Reagent-4/8 Plex multiplex labeling kit (AB Sciex LLC, Framingham, MA, USA). The labeled peptides from each group were mixed and graded using an Agilent 1260 Infinity II HPLC system (Thermo Fisher Scientific, Waltham, MA, USA). Then, an Easy nLC system (Thermo Fisher Scientific) and a Q-Exactive mass spectrometer (Thermo Fisher Scientific) were employed for liquid-phase separation and mass spectrometric separation, respectively.

Differential Protein Identification and Bioinformatic Analysis

Mascot 2.5 (Matrix Science Co., Ltd., London, UK) and Proteome Discoverer 2.1 (Max Planck Institute of Biochemistry, Martinsried, Germany) were utilized for library identification and quantitative analysis. Differentially expressed proteins were subjected to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG)enrichment analyses and passed Fisher's exact-probability testing to compare each GO classification or KEGG pathway for the target protein set and overall protein-set distribution.

Western Blotting (WB)

Total liver protein was extracted using RIPA protein lysis buffer according to the vendor's protocol (Beyotime Institute of Biotechnology) and the protein concentration was determined with a BCA protein assay kit. Proteins were loaded on 10% SDS gels, resolved using SDS-PAGE, and then transferred to Polyvinylidene

Fluoride (PVDF) membranes. Block PVDF membranes with 5% skim milk for 2 h. After blocking, primary antibodies against CacyBP/SIP (1:1000, ab720326, Abcam), CCND1 (1:1000, ab263808, Abcam), c-Myc (1:1,000, ab32072, Abcam), GSK3β (1:1,000, ab32391, Abcam), APC (1:1,000, ab40778, Abcam) and β-catenin (1:5,000, ab32572, Abcam) were added and incubated overnight at 4°C. Then, add a secondary antibody (IgG, 1:10 000, Abcam) and incubate at room temperature for 1 h. Signals were visualized using an enhanced chemiluminescence reagent (Thermo Fisher Scientific) and the developed film was scanned using a gel-imaging system (Ketagalan GL, WEALTEC, Nevada, USA) and analyzed with ImageJ, version 1.8.0 (National Institutes of Health, Bethesda, MD, USA).

Real-Time Quantitative–Polymerase Chain Reaction (RT-qPCR)

All gene primers reflected the GenBank database and were designed and synthesized by Shanghai Sheng Gong Biological Co., Ltd (primer sequences are listed in Table 1). Liver-tissue fat was collected and total RNA was extracted with phenol-chloroform-extracted TRIzol. RNA integrity was checked by 8% gel electrophoresis and RNA purity was determined by a 260/280 spectrophotometry ratio. RNA was reverse transcribed to cDNA using the TIAN Script II cDNA First-Strand Synthesis Kit on the base of the supplier's protocol (Prime Script TMRT reagent Kit, Takara, Japan). RT-qPCR was performed in a final volume of 20 μL by using 1 to 10 ng cDNA, 1 × SYBR Green PCR Master Mix (TaKaRa), 0.5 μm each sense and antisense primers or 0.5 μm each GAPDH primers (Table 1). The experiment was repeated three times. Relative mRNA levels were calculated using the $2^{-\Delta\Delta}$CT method (Livak and Schmittgen, 2001), and GAPDH was selected as the internal reference gene.

DNA Methylation

Genomic DNA was extracted from liver tissue according to the instructions of the TIAN amp DNA extraction kit (Tiangen Biotech Co., Ltd., Beijing, China). Qubit was used to quantify genomic DNA and performed electrophoresis to determine DNA quality. After DNA quantification and quality inspection, an MSPI (Thermo Fisher Scientific) digestion reaction was performed and a PCR cleaning kit (Axygen, Inc., Corning, NY, USA) was used for purification. Then, 30 μL of the eluted DNA was aliquoted for end-repair and the mix was directly added (Klenow exo-, 0.5 μL; buffer, 1 μL; dNTP, 4 μL; sterile distilled water, 4.5 μL) to the overall reaction mixture and each sample was amplified using PCR. Thereafter, the nucleotides were sequenced using an Illumina genome analyzer (Illumina HiSeq X10, San Diego, CA, USA) and the filtered high-quality sequencing data were compared with rat reference-genome sequences using Bismark software (v 0.7.4) to analyze and determine abnormally methylated genes. The screening was performed with $P<0.05$ as the significance criterion. Using an Axygen PCR cleaning kit for purification, 20 μL of eluted DNA was added to methylated adapters (linker sequence information is provided in Table 1). A TIANamp DNA Extraction Kit (Tiangen Biotech Co., Ltd.) was exploited to extract whole genomic DNA. After quality control was completed gene bisulfite methylation tests were performed and 15 μL from each sample were collected according to the bisulfite DNA modification kit instructions for sulfite hydrogen salt modification and purification. Primer information was as follows.

Table 1: Primer sequences used in the present study

Genes	Sequences
C-myc	
F:	5'- GAAACGGCGAGAACAGTTGA-3'
R:	5'- CCAAGGTTGTGAGGTTGAGCAGC-3'
GAPDH	
F:	5'- GCAAGTTCAACGGCACAGT-3'
R:	5'- GCCAGTAGACTCCACGACAT-3'
GSK3β	
F:	5'- TACCACTCAAGAACTGTCAAG-3'
R:	5'- CATTAGTATCTGAGGCTGCTG-3'
β-catenin	
F:	5'TGGCTCTGGGCGCAGCGGGAGCTACC-3'
R:	5'-CGGCAAGACAGACCTTCTCCACAGTAAC-3'
CCNDI	
F:	5'- AGGAGCTGCTGCAAATGGAA-3'
R:	5'- GAACTTCACATCTGTGGCAC-3'
APC	
F:	5'- AAGCGGAGAGGTCATCTCAG-3'
R:	5'- GCTGGACTCTGACCACTACT-3'
Adapter PE1	5'-ACACTCTTCCCTACACGACGCTCTTCCGATCT-3'
AdapterPE2	5'-P-GATCGGAAGAGCACACGTCTGAACTCCAGTCAC-3'

iPE1:AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT;iPE2:CAAGCAGAAGACGGCATACGAGATAGGAATGTGACTGGAGTTCAGACGTGTGCTCTTC. After conventional PCR amplification, 15 μL of the product was transferred to a 1% agarose gel and constant-pressure electrophoresis was conducted at 105 V for 1–1.5 h. Then, the methylated and unmethylated products were selected, the gel was cut and an ABI 3730 DNA sequencing platform was used to implement sequencing.

Statistical Analysis

All data were analyzed and presented as the mean ± standard deviation using SPSS 19.0 statistical software. Comparison between groups was performed using a one-way ANOVA followed by a posthoc Tukey's test.

Results

Establishment of a Rat Model of Obesity and Hypoxic Exercise and Results of Biochemical Indices

After eight weeks, the mean rat body weight in the obesity-model group was 20% higher than in the control group; serum TC, TG, and LDL-C were significantly elevated in the model group; and HDL-C was significantly reduced (Fig. 1). We thus obtained 50 nutritionally obese rats. The mean bodyweight of the C group rats was significantly higher than that of the CE group and highly significant relative to that of the HE group. Lee's index of the C group was significantly higher than that of the H, CE, and HE groups; and Lee's index of the H group was significantly lower than that of the CE group (Fig. 2A). The body-fat ratio of the C group was significantly higher than that of the H group, CE group, and HE group; while the body-fat ratio of the CE group was significantly higher than that of the HE group (Fig. 2A). TC and TGs in the C group were higher than in the H, CE, and HE groups; and TC in the CE and HE groups was significantly lower than in the H group. The HDL-C of the C group was significantly lower than that of the H group, CE group, and HE group; and the HDL-C of the HE group was significantly higher than that of the H group. LDL-C in the C group was significantly elevated relative to that in the H group and highly significant compared with that in the CE and HE groups. We also observed that HDL in the HE group was significantly lower than in the H group (Fig. 2C). Of all the experimental regimens, hypoxic exercise yielded the most robust effects for the specified parameters.

Proteomic Analyses of the Normoxic Quiet (C) and Hypoxic Quiet (H) Groups

We executed iTRAQ proteomics to analyze peptides from the C and H groups. We employed the C and H groups for reference using the *Rattus norvegicus* database and identified the following: 92,565 Secondary mass spectra; 42,927 peptides; and 4,545 proteins. Differentially expressed proteins were defined as those that met the screening criteria of expression differences >1.2-fold and with $P<0.05$. Further analysis revealed significant differences in the expression of 70 proteins between the C and H groups and of these, 33 proteins were up-regulated and 37 down-regulated.

Proteomic Results of the CE Group and HE Group

The CE and HE groups were applied as references using the Rattus norvegicus database, which identified the following: 87,384 Secondary mass spectra; 42,003 peptides; and 4,433 proteins. Differentially expressed proteins were defined as those that met the screening criteria of expression differences >1.2-fold and with $P<0.05$. We found 110 significantly differentially expressed proteins in the HE and CE groups, of which 61 proteins were up-regulated and 49 proteins were down-regulated.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Analyses

Based on iTRAQ proteomic screening, we next conducted GO-enrichment analysis of differentially expressed proteins and determined their associated biological processes, molecular functions, and cellular components (Zhu *et al*., 2014)-as well as the properties of their corresponding genes and gene products. The top 30 proteins that were significantly enriched in the HE and CE groups were found to be associated with nuclease activity, positive regulation of ribozyme complex metabolism, and nuclear transcription of mRNA decomposition-process proteins (Fig. 3A). The top 30 proteins were also highly enriched in the H and C groups and associated with positive regulation of cell growth, ultra-long chain fatty acid coenzyme activity, long-chain fatty acid coenzyme activity, fatty acid ligase activity, and lipid transport activity (Fig. 3B). Additionally, we found 11 significantly enriched KEGG pathways in the HE and CE groups, which included those involving drug metabolism-cytochrome P450, and cytochrome P450 metabolism of xenogeneic drugs, Hypoxia-Inducible Factor-1 (HIF-1) signaling pathway, and Wnt signaling pathway (Fig. 3C). We found 14 significantly enriched KEGG pathways in the H and C groups-including insulin resistance, PPAR signaling pathway, insulin secretion, and other signaling pathways (Fig. 3D).

WB Analysis to Determine Changes in CacyBP/SIP Protein Expression

To determine changes in the expression of CalCyclin (S100A6)-binding protein (CacyBP/SIP), we implemented WB analysis and showed that the protein

expression of CacyBP/SIP in the HE group was significantly lower than in the CE, H, and C groups; we noted no differences in any other groups (Fig. 4A). Furthermore, protein expression levels of c-myc and β-catenin (which are associated with the Wnt/β-catenin-signaling pathway) in the HE group were significantly elevated relative to levels in the CE group (Fig. 4B, 4C).

Changes in the Levels of mRNAs and Proteins Related to Wnt/β-catenin Signaling

Results of real-time PCR revealed that c-myc and β-catenin mRNA levels were significantly augmented in the HE group compared with levels in the CE group (Fig. 4), whereas GSK3β and APC mRNA levels were significantly attenuated (Fig. 4). In contrast, there was no difference in CCND1 mRNA levels between the HE and NE groups (Fig. 5).

Changes in the Methylation Sites of Genes Related to Wnt Signaling

The results of methylation analysis of CpG promoter sites of genes related to the Wnt-signaling pathway showed that the difference in Fzd2 methylation between the HE and CE groups was -18.2% and that in Wnt2b methylation was -7.6%. In addition, molecules associated with the Wnt-signaling pathway (Sox2 and Sox7), as well as the Wnt-signaling pathway antagonists Sfrp1 and Sfrp2, also exhibited methylation differences between the HE and CE groups. Furthermore, genes with significant methylation at CpG promoter sites related to Wnt signaling included the following: Fzd2, Fzd9, Wnt16, Wnt2b, Sfrp1, Sfrp2, Sox2, and Sox7 (Table 2).

The results of Differentially Methylated Regions (DMRs) in molecules associated with the Wnt-signaling pathway showed that differences between the HE and NE groups of 21.7% for Cdc42bp, 6.38% for Fzd10, 2.59% for Fzd1, 3.3% for Gsk-3β, −8.22% for Wnt16 and 4.7% for Wnt6. The significant DMRs between the HE and ME groups were as follows: Cdc, Fzd10, Gsk-3β, Fzd1, Wnt16, and Wnt6 (Table 3). We also analyzed the DMRs in regions associated with other pathways related to Wnt signaling and demonstrated differences between the HE and CE groups of 22.9% for Pik3cd, 3.1% for PPARα, and 1.98% for Hiflα (Table 4).

Table 2: CpG promoter sites of Wnt signaling pathway related genes were methylated in the HE group and CE group

Pvalue	Qvalue	Meth. diff	Gene
7.79E-191	3.76E-189	62.839911530	Wnt16
1.96E-07	5.17E-070	-7.611353456	Wnt2b
2.12E-239	1.87E-237	-92.520775620	Fzd2
8.81E-35	4.78E-340	-18.289087500	Fzd9
9.85E-79	1.94E-780	-5.074673586	Sfrp2
1.20E-205	1.08E-204	-5.223717078	Sfrp1
6.14E-62	9.00E-620	8.127329700	Sox2
3.59E-08	1.52E-080	-4.194371749	Sox7

Table 3: DMR methylation information of Wnt signaling pathway difference region between HE group and CE group

Pvalue	Qvalue	Meth. diff	Gene
2.36E-169	1.60E-168	21.712325330	Cdc42bpg
2.96E-37	2.72E-370	2.594122537	Fzd1
3.59E-224	3.96E-223	6.381529274	Fzd10
7.00E-38	6.52E-380	3.302817024	Gsk3β
5.80E-14	2.99E-140	0.751253394	Lrp5
9.46E-40	9.18E-400	-8.228840125	Wnt16
2.54E-50	3.02E-500	4.717568915	Wnt6
1.27E-35	1.12E-350	1.892110700	Wnt9b

Table 4: DMR methylation information in the different regions of genes related to the Wnt signaling pathway between the HE group and CE group

P-value	Qvalue	Meth. diff	Gene
3.87E-20	2.40E-200	1.981438164	Hif1α
1.44E-132	6.14E-132	22.90272208	Pik3cd
2.06E-48	2.36E-480	3.095007241	PPARα

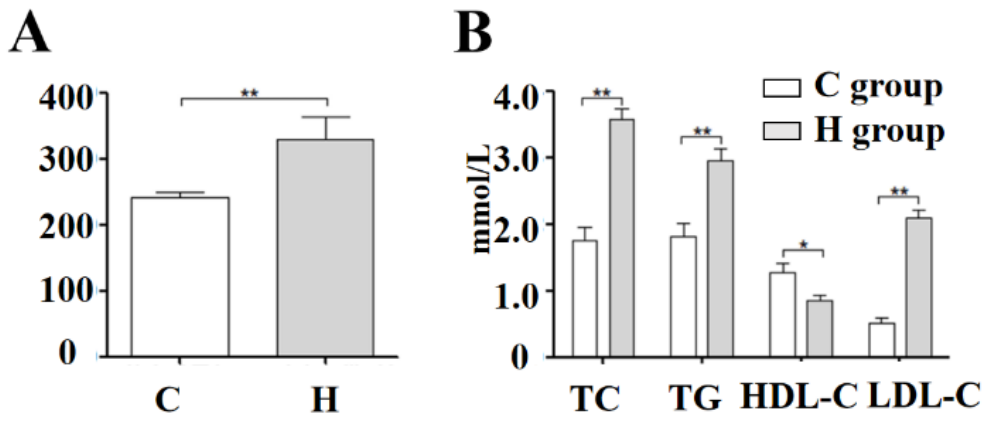


Fig. 1: Establishment of a rat model of obesity. (A) Changes in body weight of rats in the common diet control group (C) and nutritional obese rat model group (H); (B) Changes in TC, TG, HDL-C, and LDL-C of rats in the common diet control group and nutritional obese rat model group

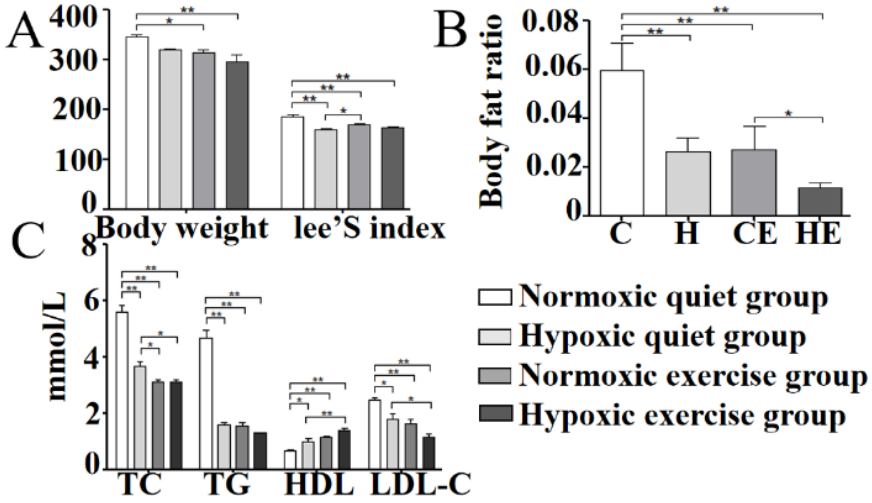
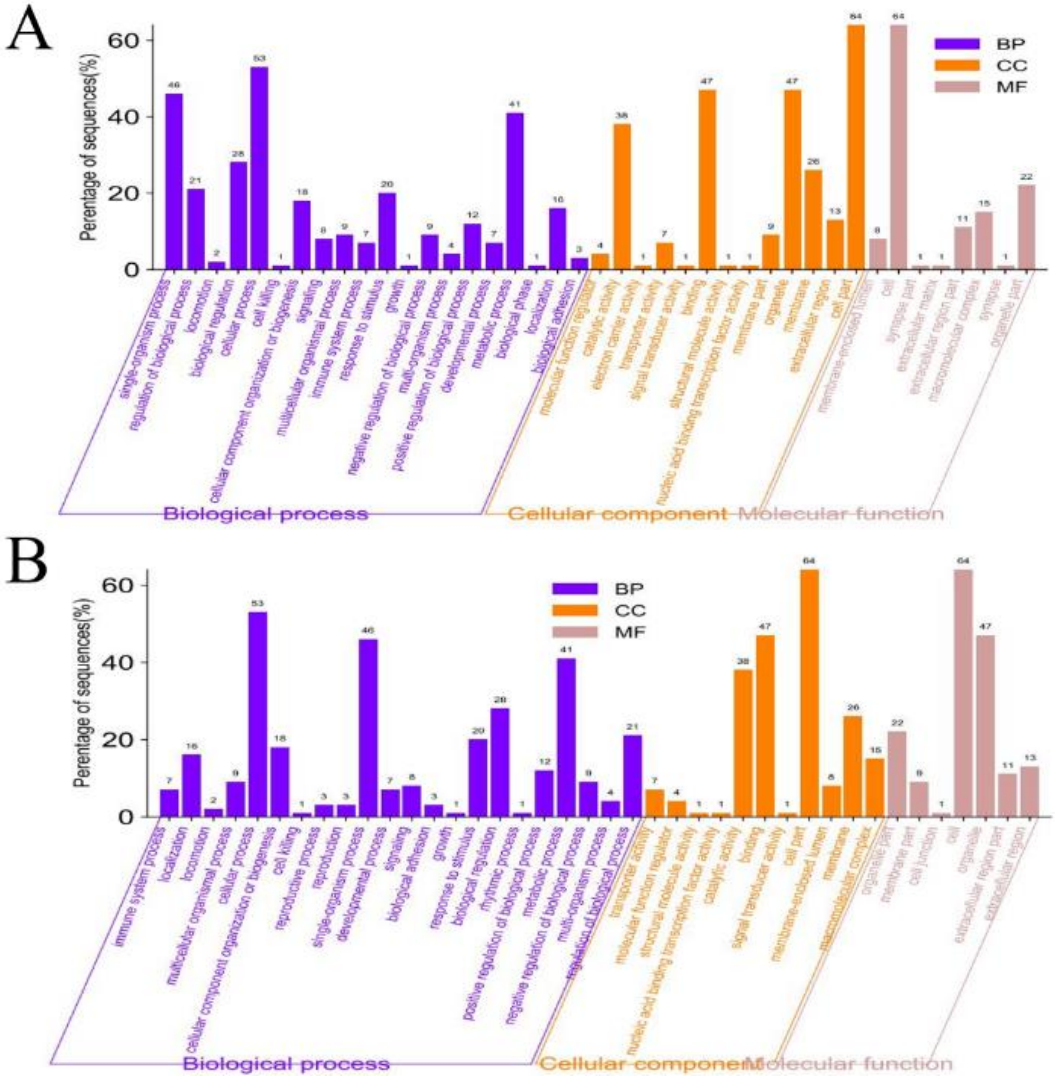


Fig. 2: Effects of experimental intervention on morphological and serum indexes of nutritional obese rats. Analysis of the effects of different exercise regimens on (A) body weight and Lee's index, (B) body fat ratio, and (C) serum index in obese rats (n = 10 per group)



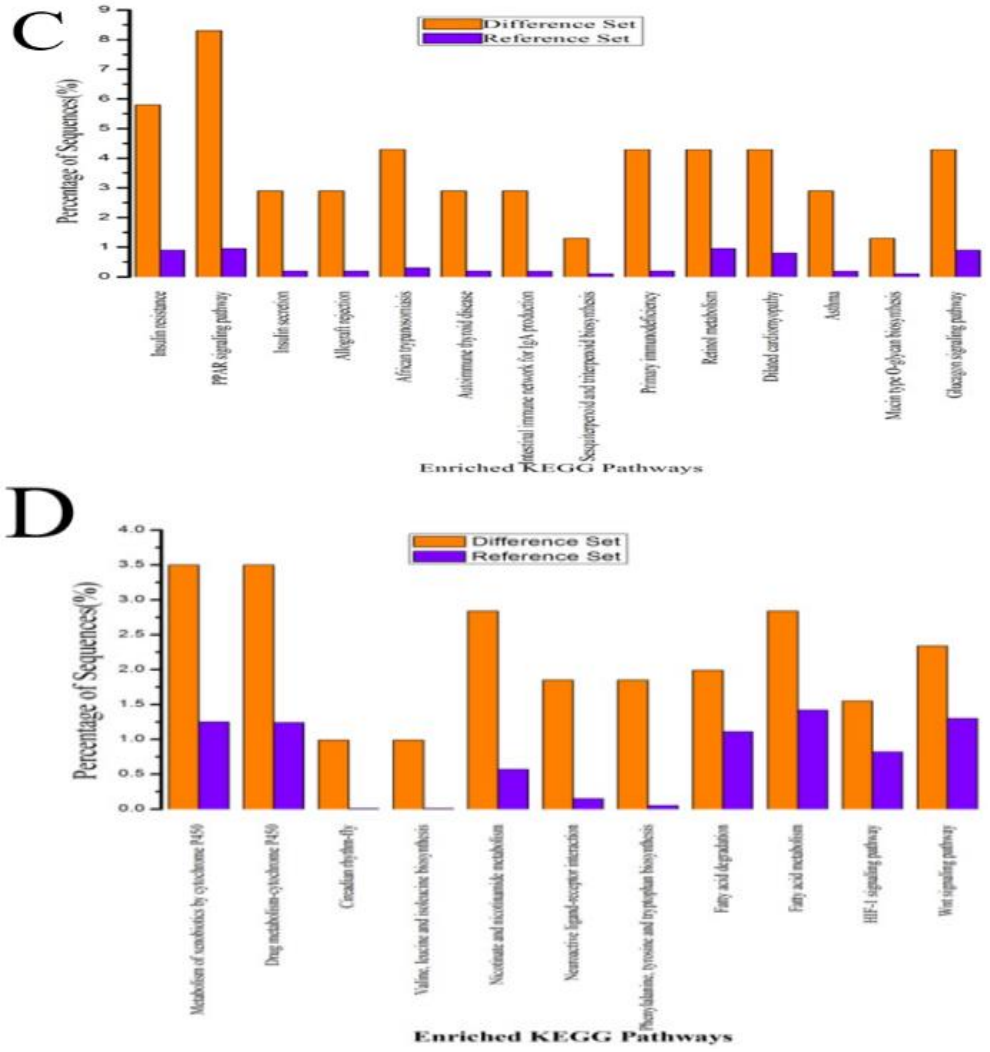


Fig. 3: GO-function enrichment and KEGG-pathway enrichment. (A) Significantly enriched GO-term statistics in the HE and CE groups. (B) Significantly enriched GO-term statistics in the H and C groups. (C) Significantly enriched KEGG pathways in the HE and CE groups. (D) Significantly enriched KEGG pathways in the H and C groups

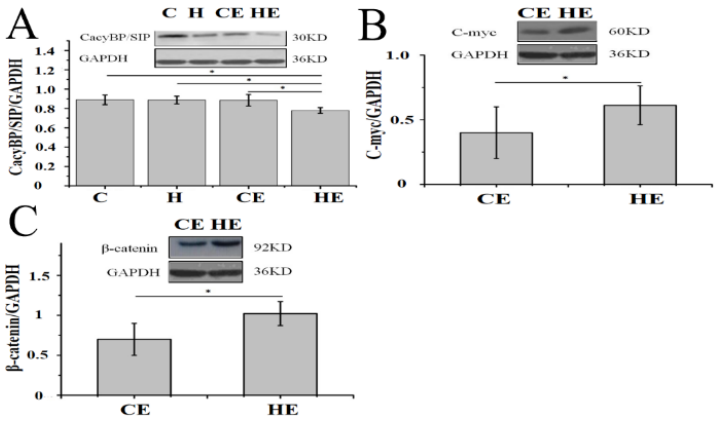


Fig. 4: Changes in the expression of Wnt/β-catenin signaling pathway molecules. (A) CacyBP/SIP protein expression. Results showed that CacyBP/SIP levels in the HE group were significantly lower than those in the CE group, H group, and C group ($P<0.05$). Changes in (B) c-myc protein expression and (C) β-catenin protein expression

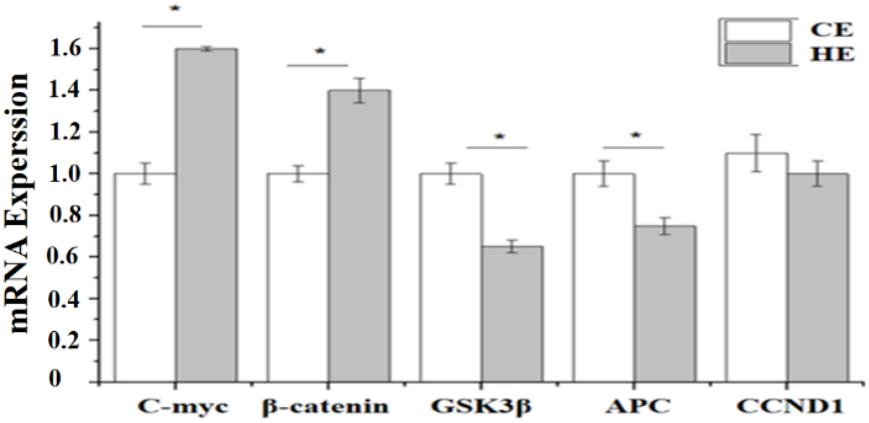


Fig. 5: Changes in mRNA levels of molecules related to the Wnt/β-catenin signaling pathway. Changes in c-myc mRNA expression, β-catenin mRNA expression, GSK3βmRNA, APC mRNA expression, and CCND1 mRNA expression

Discussion

The results of our study indicated that compared with the normoxic Exercise (CE) and control groups, rats in the HE group experienced a significant diminution in body fat. Moderate physical training can effectively promote the degradation of body fat and some studies found that the effects of altitude/hypoxic exercise on body weight and body fat were more significant than for normoxic exercise (Wee and Climstein, 2015). Exercise in a hypoxic environment can help reduce weight and fat (Tobias *et al*., 2019). Exercise promotes energy expenditure, further preventing energy from being stored as fat (Cheng *et al*., 2020).

The Wnt/β-catenin-signaling pathway is the primary negative regulator of adipocyte differentiation (Ross *et al*., 2000), and research has shown that long-term moderate exercise can activate the Wnt pathway (Bayod *et al*., 2014) and that hypoxic exercise also influences Wnt/β-catenin signaling (Strillacci *et al*., 2013). CacyBP/SIP is a member of the ubiquitin ligase complex that is involved in β-catenin ubiquitination and degradation (Anna *et al*., 2016). In the present study, we ascertained that the Wnt-signaling pathway underwent significant changes in the HE group compared with the CE group; quantitative protein analysis revealed that CACYBP/SIP (protein code Q6AYK6) in the HE group/CE group was 0.83 and that protein levels were significantly reduced. After GO-enrichment analysis and KEGG database signal-pathway, analysis-as well as the analysis of CacyBP/SIP during hypoxic exercise-we determined that the Wnt/β-catenin-signaling pathway was activated in obese rats participating in the hypoxic exercise. Therefore, CacyBP/SIP may be involved in the Wnt-signaling pathway and thus affect the formation of fat in a hypoxic environment.

The Wnt-signaling pathway is involved primarily in cellular proliferation, differentiation, polarization, and apoptosis during embryonic development in mammals (Sun *et al*., 2020). And because it can transmit signals that induce cellular proliferation or differentiation in cells and tissues, Wnt has attracted considerable attention in research. β-catenin expression activates Wnt-catenin signaling and the regulation of Wnt signaling is closely related to the activity of β-catenin (Clevers and Nusse, 2012). In this study, we compared the HE and CE groups and observed the mRNA and protein levels for the downstream effector molecules c-myc and β-catenin of the Wnt/β-catenin-signaling pathway significantly elevated; however, GSK3β and APC mRNA levels exhibited significantly attenuated expression. GSK3β, APC, actin, and axin combine to form a polyprotein complex with reduced activity; we also noted that the composite molecule's inhibitory effect on the phosphorylation of β-catenin was reduced, thereby augmenting the expression of β-catenin in the cytoplasm and translocating accumulated β-catenin into the nucleus. In the nucleus, β-catenin binds to the transcription factor LEF/TCF to activate the expression of downstream genes. Previous reports have focused on 3T3-L1 preadipocyte lines or mouse and human primary preadipocytes in vitro and there are relatively few extant studies addressing the effects of the Wnt/β-catenin-signaling pathway on obese rats in vivo in an environment of hypoxic exercise.

Our subsequent experimental results also confirmed these data. As such, exercise exerts a significant effect on DNA methylation in different tissues (Grazioli *et al*., 2017) and after exercise, genes that change methylation include those with energy metabolism, muscle regeneration, and inflammation (Barrès *et al*., 2012, Bryan *et al*., 2013, Jeoung and Harris, 2008). One of the causes of aberrant activation of Wnt/β-catenin signaling in human cancer is epigenetic silencing of Wnt regulators. And negative regulators of Wnt include intracellular Wnt antagonists (e.g., APC and DACT), extracellular Wnt inhibitors (e.g., SFRP, WIF, and DKK), non-transformed

Wnt ligands (e.g., WntSA and Wnt7A), certain nuclear proteins (SOX) and E-cadherin (Cruciat and Niehrs, 2013, Eubelen *et al*., 2018, Krishnamurthy and Kurzrock, 2018). In the present study, we screened a total of 675 single CpG-methylation sites with methylation increases or decreases of >25% in the HE group compared with the CE group. We observed that there were 213 differentially expressed genes with DMRs in their promoters and CDSs; the average methylation of all CpGs was 1.1% higher in the HE group (e.g., in G33c, G12a, G33d) relative to the CE group (e.g., in G26a, G35e, G31b). Genes related to the Wnt-signaling pathway were screened in this experiment and genes with sites showing significant methylation at their CpG promoter included Fzd2, Fzd9, Wnt16, Wnt2b, Sfrp1, Sfrp2, Sox1, and Sox2. Furthermore, DMRs included those in Cdc, Fzd10, Gsk-3β, PI3k, Wnt16, and Wnt6 genes (Tian *et al*., 2019). These findings suggested that changes in Wnt/β-catenin signaling may represent one of the mechanisms underlying weight reduction in the HE group. Similarly, gene silencing caused by abnormal methylation in the promoter region of Wnt genes may also lead to anomalies in Wnt/β-catenin signaling. Furthermore, we hypothesized that methylation of the promoter regions of genes-including genes in the Wnt/β-catenin-signaling pathway may have been involved in the onset and development of weight loss that we observed in the HE group in the current study.

Conclusion

In this study, we exploited the iTRAQ proteomics technique to identify and screen different proteins and ascertained that the protein abundance of CacyBP/SIP decreased after hypoxic exercise. After GO-enrichment and KEGG database signal-pathway, analyses as well as that of CacyBP/SIP during hypoxic exercise, we determined that the Wnt/β-catenin-signaling pathway was activated in obese rats with hypoxic exercise. By measuring the mRNA transcription levels of several key factors in the Wnt-signaling pathway, we obtained the same results as with our iTRAQ proteomics analysis. The Wnt/β-catenin-signaling pathway was thus activated in obese rats undergoing hypoxic exercise and stimulated the expression of key adipose-gene transcription factors, thereby participating in the processes of fat formation and metabolism in hypoxic obese rats.

Acknowledgment

The Taishan Scholars Program of Shandong Province (tsqn201909148) and the Shandong Province Key R&D Program (2020CXGC010902) provided financial support for this research.

Author's Contributions

Peiming Xu, Qinglu Wang, and Xuewen Tian: Participate in article writing, design, and supervising project completion.

Zhiyuan Sun: Article conception and writing.

Xiaoyi Tian: Data Collection.

Yingjie Yang: Design and supervise the completion of the project.

Peiming Xu and Zhiyuan Sun: Contributed equally to this study.

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