Short-chain fatty acid-Butyrate ameliorates hypoxia-induced pulmonary hypertension in rat

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Research article

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Abstract

Background: Gut Microbiome can produce numerous metabolites which may be absorbed into the circulation and play a critical role in the development of diseases. Our study wants to find out the roles of faecal microbiota and its metabolite in the development of pulmonary hypertension.

Methods: SD rats were expose to either normoxia (Control, n=8) or chronic hypoxia (10% O 2) (n=16). Rats in chronic hypoxia were randomly received sodium butyrate (500 mg/kg) (n=8) or volume-matched saline (n=8) once a day. Rats in normoxia condition also received volume-matched saline once a day. RVSP and lung vascular features were assessed after 3 week's treatment. Rats' fecal samples were collected and analyzed by GCMS and 6S/18S Amplicon Sequencing. Activation of HIF-1α, HMGB2, TLR4, TNF-α, HDAC5 and PCNA was assessed using Western blot analysis. The effect of sodium butyrate on proliferation of pulmonary artery smooth muscle cell, induced by PDGF or hypoxia (1% O 2), was evaluated by CCK-8, EDU assay and wound healing assay.

Results: Hypoxia significantly increased RVSP and induced a significant decrease in butyrate-producing bacteria, but a slight decrease of SCFAs without statistic difference. Administration of sodium butyrate remarkably attenuated the RVSP and improved lung vascular features. Decreased expression of HDAC5, PCNA, HIF-1α, HMGB2, TLR4, TNF-α and inhibited proliferation of PASMC were found in rats or cells with sodium butyrate.

Conclusions: Decreased butyrate-producing bacteria may contribute to the development of PH, and sodium butyrate supplement can effectively attenuate hypoxia-induced PH in rats probably by inhibiting the proliferation and migration of PASMC.

Background

Pulmonary hypertension (PH) is a considerable worldwide health problem. According to recent estimates, the prevalence of pulmonary hypertension is about 1% of the global population. When it comes to individuals aged over 65 years old, the number increases to 10%. In addition, approximately 80% of pulmonary hypertension patients are living in developing countries[1]. It is meaningful and urgent to clarify the pathogenesis of PH. Previously, vasoconstriction was believed to act a vital role in PH pathogenesis. However, now it is more likely to believe that pulmonary vascular remodeling, characterized by uncontrolled proliferation and resistance to apoptosis of PASMC and pulmonary artery endothelial cells (PAEC), is the pivotal causal factor of PH[2]. PASMC has been proved to play an important role in the development of various types of pulmonary hypertension. Different mechanisms can act on apoptosis, hypoxia induced factor (HIF), histone deacetylase (HDAC) and inflammation, leading to uncontrolled proliferation of PASMC, and finally resulting in pulmonary hypertension[3,4].

Intestinal microbiota has been widely studied in many health problems, including cardiovascular diseases[5]. But little is known about the connection between intestinal microbiota and PH. Short-chain

fatty acids (SCFAs) are the major fermented products of intestinal microbiota, especially acetate, propionate, and butyrate[6]. By analyzing Meta-genomic Data, Marius Vital et al. found that Genomes of 225 bacteria have the potential to produce butyrate, including many previously unknown candidates[7]. Most candidates are in distinct families within the Firmicutes, but members of other nine phyla, such as Actinobacteria, Bacteroidetes, Fusobacteria, Proteobacteria, Spirochaetes, and Thermotogae, were also recognized as potential butyrate producers. Duncan et al and Louis et al. stated that 8 kinds of microbes, Coprococcus comes, Coprococcus eutactus, Anaerostipes spp, Coprococcus catus, Eubacterium rectale, Eubacterium hallii, Faecalibacterium prausnitzii, Roseburia spp take the major responsibility for producing butyrate and they all are members of Firmicutes[8,9]. Callejo et al. reported that hypoxia-induced PH rats showed gut dysbiosis and among the Bacteroidetes phylum, all families were decreased in hypoxia-induced PH rats[10]. Our preliminary study using MCT-induced PH rat models revealed decreased number of butyrate-producing bacteria and SCFAs. Among all SCFAs, butyrate is the most widely studied and testified to protect form cancer and inflammation by influencing cell proliferation and apoptosis, which maybe results from inhibiting HDACs[11,12]. Cantoni S et al. showed that sodium butyrate inhibited PASMCs proliferation and migration induced by PDGF[13]. In addition, Zhang et al. clarified that sodium butyrate significantly decreased the lethality of severe sepsis in rats by inhibiting the expression of high-mobility group box (HMGB) protein and TNF-α[14].

Therefore, it is important to uncover the gut microbiome changes in PH and we wonder whether sodium butyrate could reverse the elevated cell proliferation, migration and inflammation of PASMC in PH, with an aim of providing more evidence for exploring novel therapeutic targets.

Methods

Animals and experimental design

24 adult male Sprague-Dawley (SD) rats (body weight around 200g) were used. The animals were purchased from Hunan Biomodel Organism Company, Hunan, China and housed in a temperature-controlled room with a 12-h light and 12-h dark-cycles in Laboratory Animal Center of Central South University. Rats were allowed free access to water and a standard laboratory diet. They were randomly divided into three groups: 1) Control Group: rats lived in normoxic environment and were administrated with normal saline (NS) by gavage once a day; 2) Hypoxia Group: rats were continuously exposed to 10% O_2 for 3 weeks and were simultaneously intragastric administration of NS once a day; 3) Hypoxia plus Sodium Butyrate Group: rats were consecutively exposed to 10% O_2 for 3 weeks, and at the same time, they were intragastric administration of sodium butyrate (500mg/kg) once a day. Animals were weighed every other day and treatment doses were calculated accordingly. The protocol was approved by the Animal Research Committee of Central South University, Hunan, China and carried out in accordance with the Guidelines for Animal Experimentation of Central South University and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication NO. 85-23, revised 2011).

RVSP measurement, tissue and faeces collection, tissue immunofluorescence

To measure rats' right ventricular systolic pressure (RVSP) levels, they were anaesthetised (10% Chloral hydrate 0.4ml/100g i.p) and a polyethylene PE50 catheter was inserted into the right external jugular vein and then threaded form the right atrium into the right ventricle (RV) to record the RVSP using BL-420F system. Then, the rats were euthanized by exsanguination. Following PBS perfusion, lung tissues and fecal samples were collected, saved in liquid nitrogen temporarily and then stored at −80°C for biochemical measurements. Parts of lung tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned. For immunofluorescence staining, the embedded tissues were cut into 5 μm-thick slices. Slices and Anti-α-smooth muscle actin (α-SMA, 1:100, ServiceBio, China) or Anti-HIF-1α (1:100, Santacruz, USA) were incubated at 4 °C overnight and then with Alexa 488 conjugated anti-rat IgG (1:200, ServiceBio, China) at room temperature for 2 hours. Finally, slices were viewed and photoed with a laser scanning confocal microscope. Relative fluorescence intensity was analyzed per lung section at a magnification of 200 using Image J software.

Cell isolation and culture

PASMC was isolated from SD rats (body weight around 150 g) with a previously described method[15]. Cells were cultured in complete medium consisting of DMEM/F12 supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 ug/mL streptomycin (all reagents from Sigma) in a standard cell incubator (37 ℃, 5% CO_2). Some of the isolated cells were seeded on a confocal plate and the next day, cells were washed with PBS, then fixed with 4% paraformaldehyde for 10 min. Cells were then permeabilized for 15min in 0.1% Triton, and following by PBS washing, cells were incubated with anti-α-smooth muscle actin (α-SMA, 1:100, ServiceBio, China) at 4 °C overnight and then with Alexa 488 conjugated anti-rat IgG (1:200, ServiceBio, China) at room temperature for 40 minutes. Finally, the cells were viewed with a fluorescence microscope to confirm that they were smooth muscle cells. For all experiments, PASMC was starved 12 hours with DMEM/F12 medium containing no serum. After 12 h, cells were induced to proliferate or migrate by replacing starvation medium with fresh DMEM/F12 medium, containing 20 ng/ml platelet-derived growth factor (PDGF) beta homodimer (PeProtech, Rocky Hill, NJ, USA) or exposed to hypoxia incubator (37 ℃,1% O_2). Concurrently, sodium butyrate was dissolved in DMEM/F12 medium and added with a final concentration of 3 mmol/L; volume matched DMEM/F12 medium was added into control group. PASMC from the third to the fifth passages was used for all studies. To evaluate the proliferation ability of PASMC, CCK-8 and EDU assay were used according to the instructions.

Western Blotting

Lung tissues were ground in ice-cold lysis buffer with 1 mM protease inhibitor cocktail (Beyotime, China), and then centrifuged at 10,000 × g for 15 min, after which the supernatants were collected. Cell protein samples were prepared and analyzed as previously described[16]. Briefly, cells were lysed with RIPA lysis buffer supplemented with 1 mM protease inhibitor cocktail (Beyotime, China). The protein concentrations were measured with the BCA Protein Assay kit (Beyotime, China). The total proteins were incubated in boiling water for 5 min. The quantity of total proteins loaded on each gel was 50 ug for lung tissues, while 20 ug for cell samples. Total proteins were separated on 8% or 10% SDS/PAGE gels. Then the proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skimmed milk in PBST for 2 hours at room temperature and incubated with specific primary antibodies in a shaker (4 ℃), overnight. β-actin (1:5000, Proteintech, USA) was used as the control. Immunoreactive bands were detected using HRP-conjugated goat anti-rabbit or anti-mouse IgG (1:5000, Proteintech, USA). Then, protein bands were detected by enhanced chemiluminescence (Thermofisher, USA) and quantified by Image J software. Primary antibodies included rabbit polyclonal antibody against HDAC5 (1:1000, Proteintech, USA), rabbit polyclonal antibody against PCNA (1:2000, Proteintech, USA), rabbit polyclonal antibody against HMGB2 (1:10000, Abcam, USA), rabbit polyclonal antibodiy against TNF-α (1:1000, ABclonal, USA), mouse monoclonal antibody against HIF-1α (1:200, Santacruz, USA), mouse monoclonal antibody against TLR4 (1:1000, Santacruz, USA).

CCK-8 assay

Cell proliferation was evaluated with the CCK-8 Kit, according to the manufacturer's instructions (Beyotime, China). Briefly, at the end of treatment, 10 ul CCK-8 solution was added to each well and then continually incubated for 2 h. Finally, Absorbance were collected at 450 nm with a multiwell plate reader (Thermofisher, USA).

EDU assay

PASMC was seeded and starved with DMEM/F12 medium for 12 hours, then cells were exposed to PDGF (20 ng/ml) or hypoxia (1% O_2), with or without sodium butyrate (3 mmol/l). After 48 hours, cells were treated with EDU for 2 hours. Then cells were washed with PBS and fixed in 4% paraformaldehyde for 10 min. Permeabilization was performed with 0.1% Triton for 15 min and specific mixed solution was added into cells for 30 min in the darkness, according to the instruction. Finally, the cells were washed with PBS again and their nuclei were labeled with 4', 6-diamidino-2-phenylindole (10 ng/mL) for 15 min and viewed with a fluorescence microscope.

Wound healing assay

For wound healing assay, confluent cells were starved for 12 hours. A 1ml micropipette tip was used to wound cell layers and the floating cells were washed away with starvation medium. Then cells were stimulated with PDGF or hypoxia in the absence or presence of sodium butyrate. Untreated cells were used as the control and outcomes were monitored and evaluated by a phase microscopy after 48 hours.

Fecal DNA extraction, sequencing and data processing

Fecal DNA, obtained from rats, was extracted using specific DNA-extraction kit (BGI, China) according to its instructions. Then the DNA samples were analyzed with 16S/18S Amplicon Sequencing Program, based on Illumina platform, Flash, Usearch, RDP classifier software and Greengene database, which generated the detailed information of intestinal microbiota in different groups.

Fecal SCFAs detection

Fecal SCFAs were measured using gas chromatography and mass spectrometry (GC-MS), as described previously[17]. In brief, internal standards were mixed with 50 mg of faeces. Samples were analyzed by using a 7890A gas chromatograph coupled with an Agilent 5975C mass selective detector (Agilent Technologies, Santa Clara, CA). A HP-5-MS (5%-diphenyl 95%-methylpolysiloxane) capillary GC column (30 m x 250 μm i.d. 2.5 μm film thickness, Agilent Technologies) was used with helium as the carrier gas at a constant flow rate of 1 mL/min. All original data were processed with Enhanced Chemstation (Agilent Technologies). The integrated areas of the SCFAs were normalized to the internal standard and quantified with a standard curve constructed from serial dilutions of SCFAs.

Statistics

Data are presented as the mean ± SD. Comparisons between multiple groups were conducted using one-way analysis of variance (ANOVA). Comparisons between two groups were conducted using Mann-Whitney t-test. In the analysis of intestinal microbiota, kruskal test was used for three groups. The difference with $P < 0.05$ (two sides) was statistically significant.

Results

1. Decreased butyrate-producing bacteria in hypoxia-induced PH rat models.

In order to clarify the relationship between intestinal microbiome and pulmonary hypertension, we collected the fecal samples from different groups, analyzed the butyrate-producing bacteria and butyrate content in them. Our data showed that Firmicutes decreased in hypoxic rat models compared to the

control group. At the level of Family, hypoxic rat models also showed decrease in Clostridiaceae and Erysipelotrichaceae, both pertaining to Firmicutes. However, there was no difference of butyrate content in 3 groups' faeces (Fig. 1).

2. Sodium Butyrate ameliorates pulmonary hypertension in hypoxic rat models along with HIF and HDAC inhibition.

Although in different groups, there was no accordant change between butyrate-producing bacteria and butyrate content, we still wonder whether extra supplement of butyrate could ameliorate the PH process induced by hypoxia. In vivo, 24 SD rats were randomly divided into three groups: control group, hypoxia group and hypoxia plus sodium butyrate group. After 3 weeks' treatments, the results of in vivo experiment showed rats exposed to chronic hypoxia developed PH characterized by significant increased RVSP level (Fig. 2) and thickening of small pulmonary arterial wall showed by immunofluorescence of α-SMA (Fig. 3). Butyrate treatment (500 mg/kg by gavage once a day) significantly attenuated RVSP level and thickness of small pulmonary arterial wall when compared to hypoxia group. In order to further investigate the reasons why extra supplement of butyrate could ameliorate PH, based on previous works and literature, we detected some important molecules that have a vital role in the PH process from the lungs of rats. As a result, sodium butyrate could effectively reverse the increased expression of HIF-1α, HMGB2, TLR4, TNF-α and HDAC5, PCNA in the lungs of hypoxia-induced rats (Fig. 4). Apart from the aspect of Western Blotting, HIF-1α immunofluorescence of rat lungs also confirmed its decreased expression in sodium butyrate group compared to hypoxia group (Fig. 5).

3. Sodium Butyrate inhibits the proliferation and migration of PASMC probably via HIF and HDAC inhibition.

Because HIF-1α/HMGB2 are highly related to cell migration, while HDAC5/PCNA are greatly connected with cell proliferation, we further tested the effects of butyrate on proliferation and migration of PASMC. The results of CCK-8 and EDU immunofluorescence all showed butyrate treatment (3 mmol/l) significantly decreased proliferation of PASMC induced by PDGF (20 ng/ml) or hypoxia (1% O_2) (Figs. 6, 7). On the other hand, Wound-healing assay manifested that sodium butyrate (3 mmol/l) inhibited the migration of PASMC, in comparison with PDGF or hypoxia (Fig. 8). In order to answer the question whether the effects of sodium butyrate are correlated with discrepant protein expressions, detected form the lungs of rats, we prepared and analyzed the protein samples of PASMC. Consistently, Western blotting outcomes also revealed opposite effect on HIF-1α, HMGB2, TLR4, TNF-α and HDAC5, PCNA expression with sodium butyrate compared to PDGF or hypoxia (Fig. 9).

Discussion

To our knowledge, the results of our study first demonstrated a decrease of butyrate-producing bacteria in hypoxia-induced PH rats, and supplementation of sodium butyrate could ameliorate hypoxia-induced PH in rat model, marked with attenuated RVSP level and thickness of small pulmonary arterial wall. Regrettably, this study didn't achieve statistically difference of sodium butyrate in different groups' faeces. It may be because of limited samples, selected faeces-collecting position or limited intervening time. As the important energy source and molecule for colon cells, extra supplement of sodium butyrate may be abundantly absorded by colon cells and exert its effects. But the exact pathway of sodium butyrate from gut to pulmonary hypertension is still worthy of investigating. In this study, we showed the decreased protein levels of HDAC5, PCNA, and HIF-1α, HMGB2, TLR4, TNF-α with sodium butyrate, compared to hypoxia or PDGF, both in rats' lungs and PASMC. Furthermore, this study proved that sodium butyrate could effectively inhibit hypoxia or PDGF-induced proliferation of PASMC, which is partly in accordance with previous study conducted by Cantoni S et al[13].

Zhao et al. reported increased HDAC1 and HDAC5 protein levels in lungs from both IPAH patients and hypoxia-induced PH rats[18]. HDAC inhibitors, valproic acid (VPA) and suberoylanilide hydroxamic acid (SAHA), prevented and reduced pulmonary hypertension of hypoxia-induced PH rats. HDAC5 belongs to class IIa HDACs, and it has long amino-terminal extensions, which act as binding sites for transcription factors and cofactors[19]. In muscle cells, class IIa HDACs can act on genes that govern cellular growth and differentiation[20,21]. Our study also found increased HDAC5 expression in rats or PASMC exposed to hypoxia and sodium butyrate could reverse the increased expression. By studying HIF-1α knock-out mice, Yu et al, and Brusselmans et al proved that increased HIF-1α play an important role in the development of pulmonary hypertension[22,23]. In addition, previous studies also found enhanced HIF-1α levels in plexiform lesions of PH patients and pulmonary vasculature of chronic hypoxia mice[24,25,26]. Compared to control, in vitro results from Malczyk et al and Veith et al, showed an over-expression of HIF-1α with enhanced PASMC proliferation[27,28]. Given the condition that activated HIF-1α could accompany with switch from aerobic to anaerobic metabolism, a phenomenon known as Warburg effect, which also could be seen in pulmonary hypertention's PASMC, sodium butyrate may have an impact on PASMC metabolism under the condition of hypoxia[25]. By studying PASMC form HIF-1α$^{+/-}$ mice, Shimoda LA discovered inhibited hypoxia-induced proliferation[29]. Several studies showed inspiring outcomes of hypoxia-induced PH by using pharmacologic inhibitors targeting HIF activity[30]. In conclusion, there is no doubt that HIF-1α acts a crucial role in the progress of PH. So it is meaningful to see decreased expression of HIF-1α treatment with sodium butyrate in our study.

Lots of growth factors, cytokines and chemokines, including PDGF, have been claimed to stimulate cancer and PH occurrence[31,32,33]. In PH patients, some studies have found elevated levels of pro-inflammatory cytokines, such as IL-6 and TNF-α, which often have a close relationship with worse clinical outcome[34,35]. HMGB2 could enhance proliferation and migration in human aortic smooth muscle cells by inducing reactive oxygen species[36]. Therefore, it is important for sodium butyrate to decrease the protein

levels of PCNA, HMGB2, TLR4 and TNF-α, which play different roles in the development of PH and PASMC proliferation.

Our study indicates an intriguing interplay among intestinal microbiome, short-chain fatty acids and pulmonary hypertension. Sodium butyrate could ameliorate hypoxia-induced PH, inhibit the proliferation and migration of PASMC, decrease the protein expressions of HIF-1α, HMGB2, TLR4, TNF-α and HDAC5, PCNA. However the overall mechanisms behind the relationship are remaining enigmatic and awaiting further clarification. Moreover, if we can get the data of butyrate-producing bacteria and SCFAs in PH patients, comparing with this study, it would be more convincing and meaningful.

Conclusion

In summary, we demonstrated that decreased butyrate-producing bacteria may contribute to the development of PH, and sodium butyrate supplement can effectively attenuate hypoxia-induced PH in rats probably by inhibiting the proliferation and migration of PASMC.

Abbreviations

SD: Sprague-Dawley

RVSP: Right ventricular systolic pressure

GCMS: Gas chromatography and mass spectrometry

PH: Pulmonary hypertension

PASMC: Pulmonary artery smooth muscle cell

PAEC: Pulmonary artery endothelial cell

HIF-1α: Hypoxia-induced factor-1α

HMGB2: High-mobility group box 2

TLR4: Toll-like receptor 4

TNF-α: Tumor necrosis factor-α

HDAC5: Histone deacetylase 5

PCNA: Proliferating cell nuclear antigen

α-SMA: α-smooth muscle actin

PDGF: Platelet-derived growth factor

CCK-8: Cell counting kit-8

EDU: 5-Ethynyl-2′-deoxyuridine

SCFAs: Short chain fatty acids

MCT: Monocrotaline

NS: Normal saline

SB: Sodium Butyrate

DAPI: 4',6-diamidino-2-phenylindole

Declarations

Ethics approval and consent to participate

The rats experiment protocol was approved by the Animal Research Committee of Central South University, Hunan, China and carried out in accordance with the Guidelines for Animal Experimentation of Central South University and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication NO. 85-23, revised 2011).

Consent for publication

All the authors declare no competing interests.

Availability of data and material

The data set during and/or analyzed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SXH, TTZ, ZWZ, YYH, ST, LT and ZFF designed the experiment. SXH, ZWZ and ZFF wrote the manuscript. SXH and TTZ performed the experiments and analyzed the data. ZWZ and ZFF helped with data analysis and revised the manuscript. All authors reviewed the manuscript. All authors have read and approved the manuscript.

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Figures

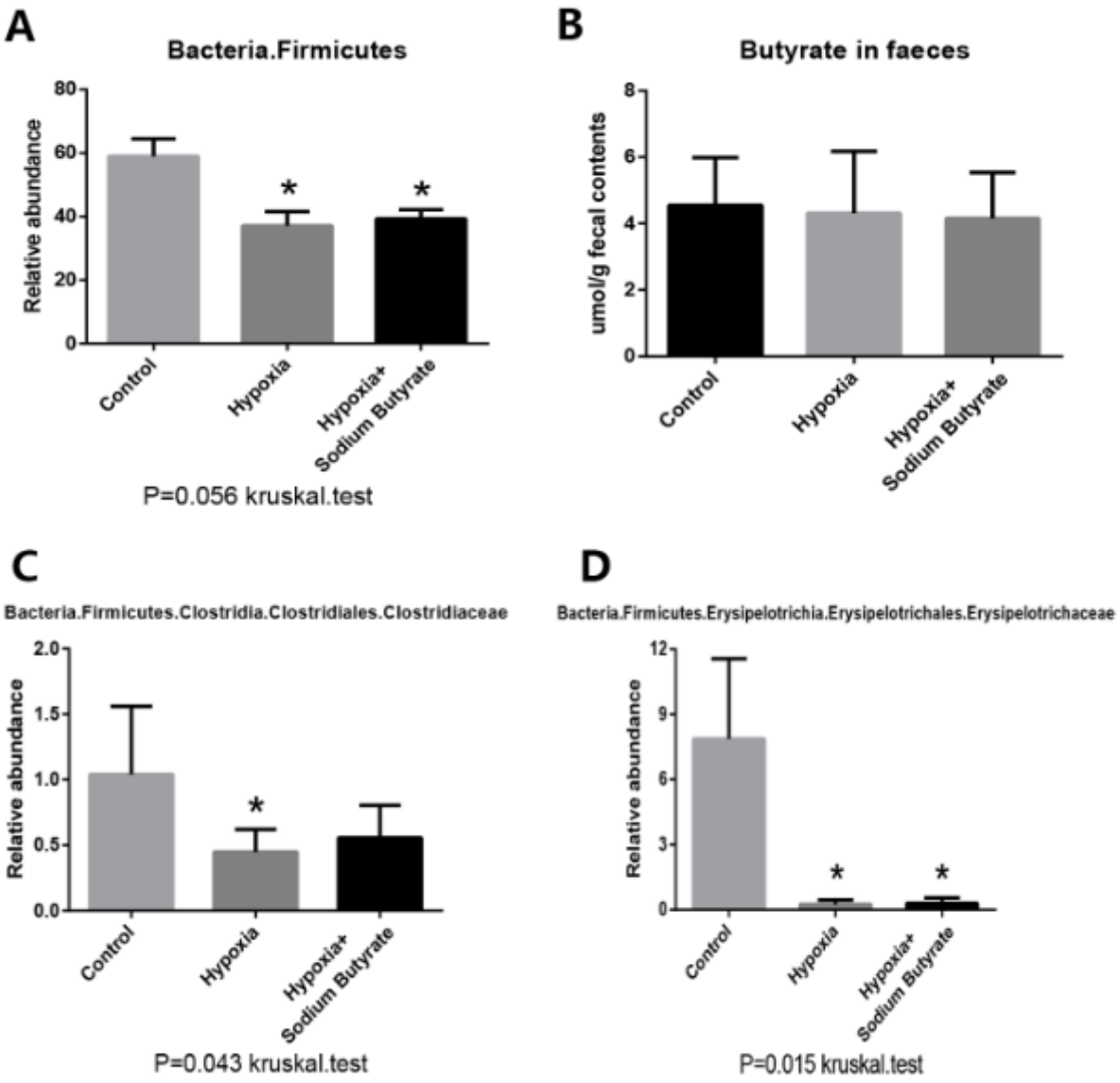

Figure 1

Analysis of butyrate-producing bacteria in faeces of rats from different groups. (A) Firmicutes (Phylum) in rats' faeces. Firmicutes decreased in hypoxia group compared to the control group. (B) Butyrate in faeces of rats. No observed statistical difference. (C) Clostridiaceae (Family) in rats' faeces. (D) Erysipelotrichaceae (Family) in rats' faeces. Clostridiaceae and Erysipelotrichaceae also decreased in hypoxia group, compared to the control group. Kruskal test was used for comparison among three groups. The data are presented as mean±SD. * $p<0.05$ compared with Control.

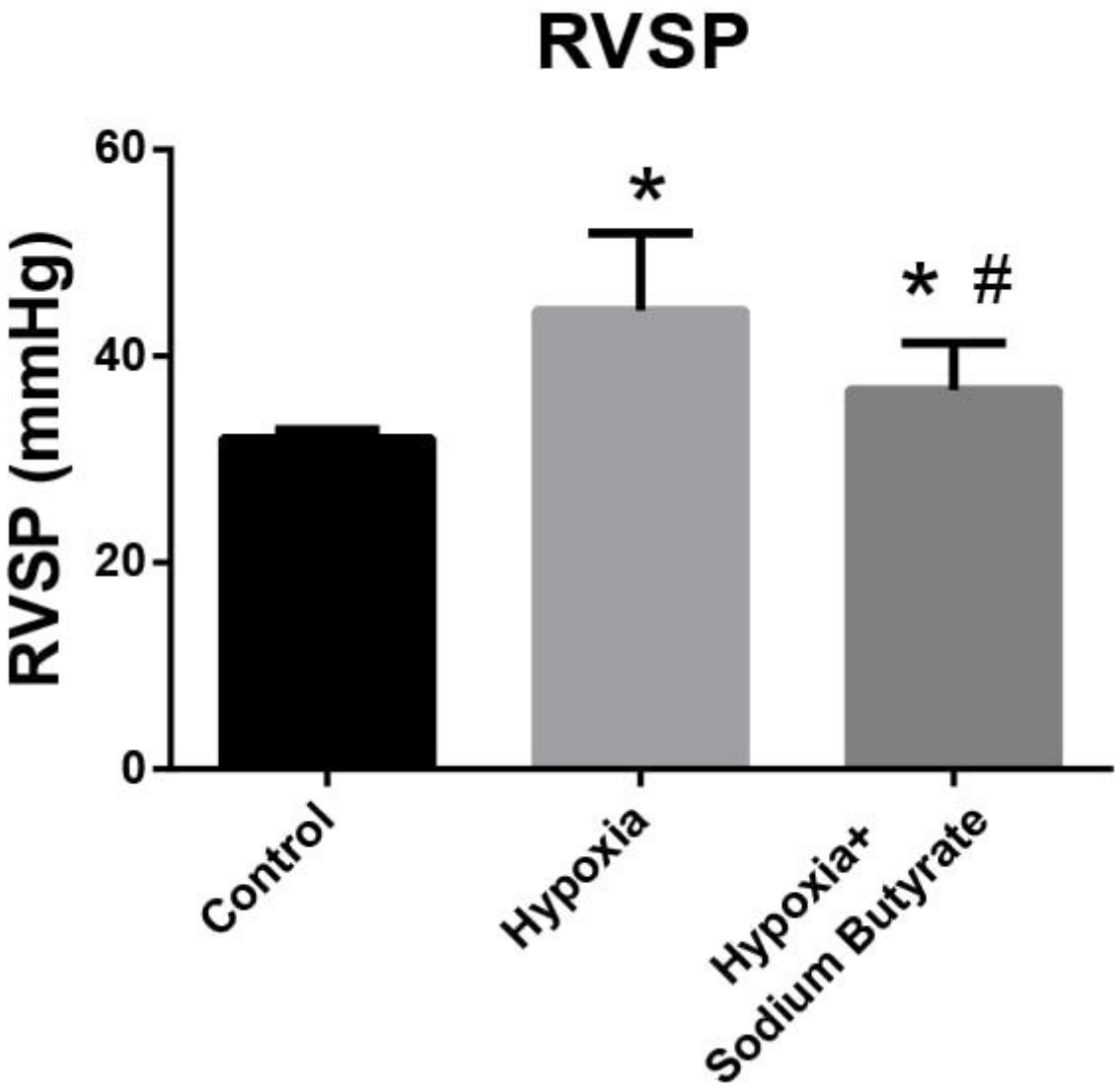

Figure 2

Right ventricular systolic pressure (RVSP) of rats. The RVSP level increased significantly in hypoxia group, compared to hypoxia + sodium butyrate and control group. The data are presented as mean±SD. * $p<0.05$ compared with Control, # $p<0.05$ compared with Hypoxia.

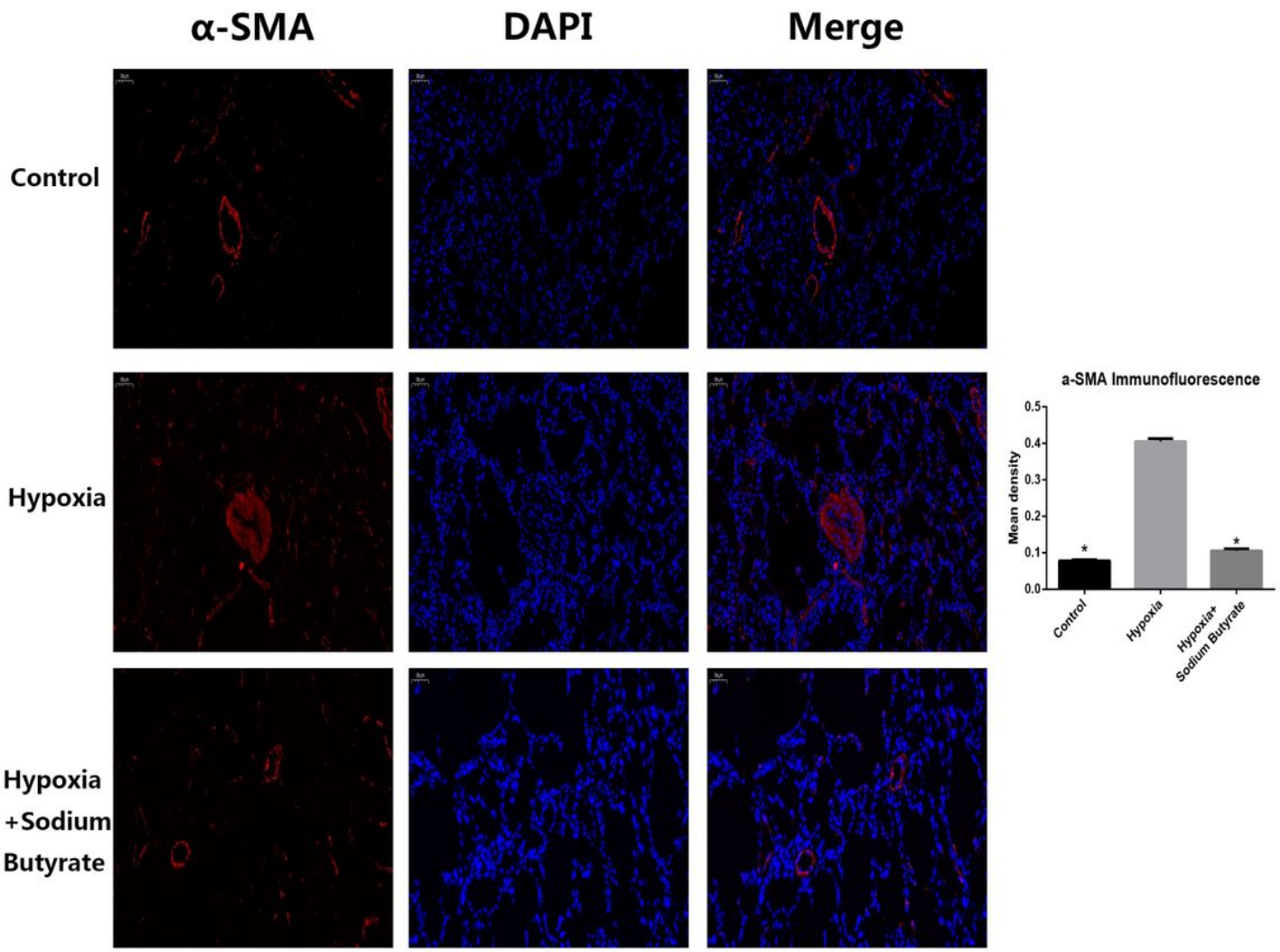

Figure 3

Immunofluorescence of α-SMA (red) in lungs of rats. Nuclei were counterstained with DAPI (blue). Compared with hypoxia + sodium butyrate and control group, the expression of α-SMA (red) in hypoxia group increased significantly. Mean density = IOD sum/AREA sum. The mean density of α-SMA are presented as mean±SD. * $p<0.05$ compared with Hypoxia. (All images are magnified at ×20)

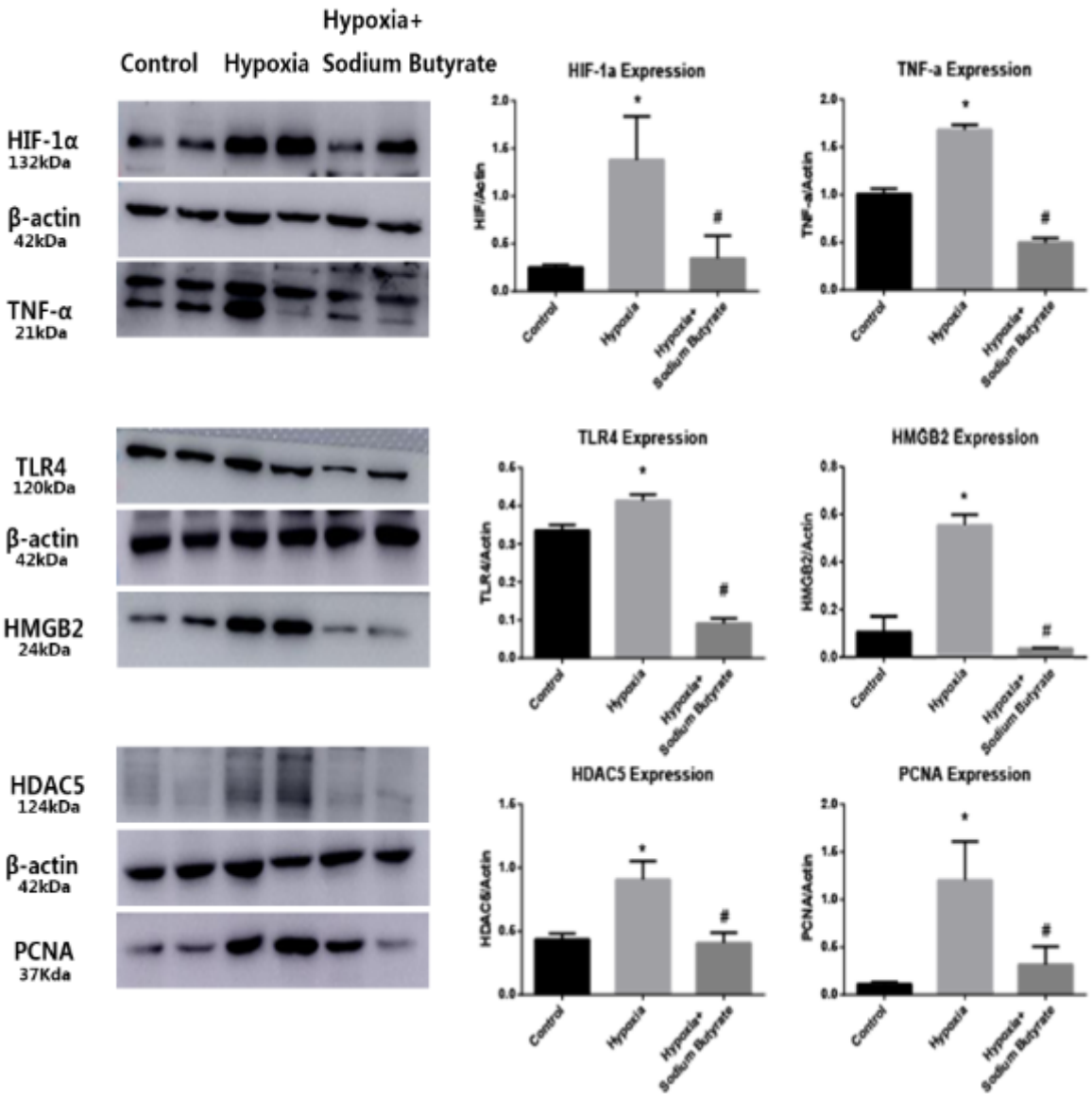

Figure 4

Western blotting of rats' lungs. HIF-1α/β-actin/TNF-α were from the same gel, TLR4/β-actin/HMGB2 were from the same gel, HDAC5/β-actin were from the same gel, while PCNA was from the different gel with same conditions. All images were automatic exposed. The expressions of HIF-1α, HMGB2, TLR4, TNF-α and HDAC5, PCNA in the lungs of hypoxia group increased, compared to hypoxia + sodium butyrate and control group. The data are presented as mean±SD. * $p<0.05$ compared with Control, # $p<0.05$ compared with Hypoxia.

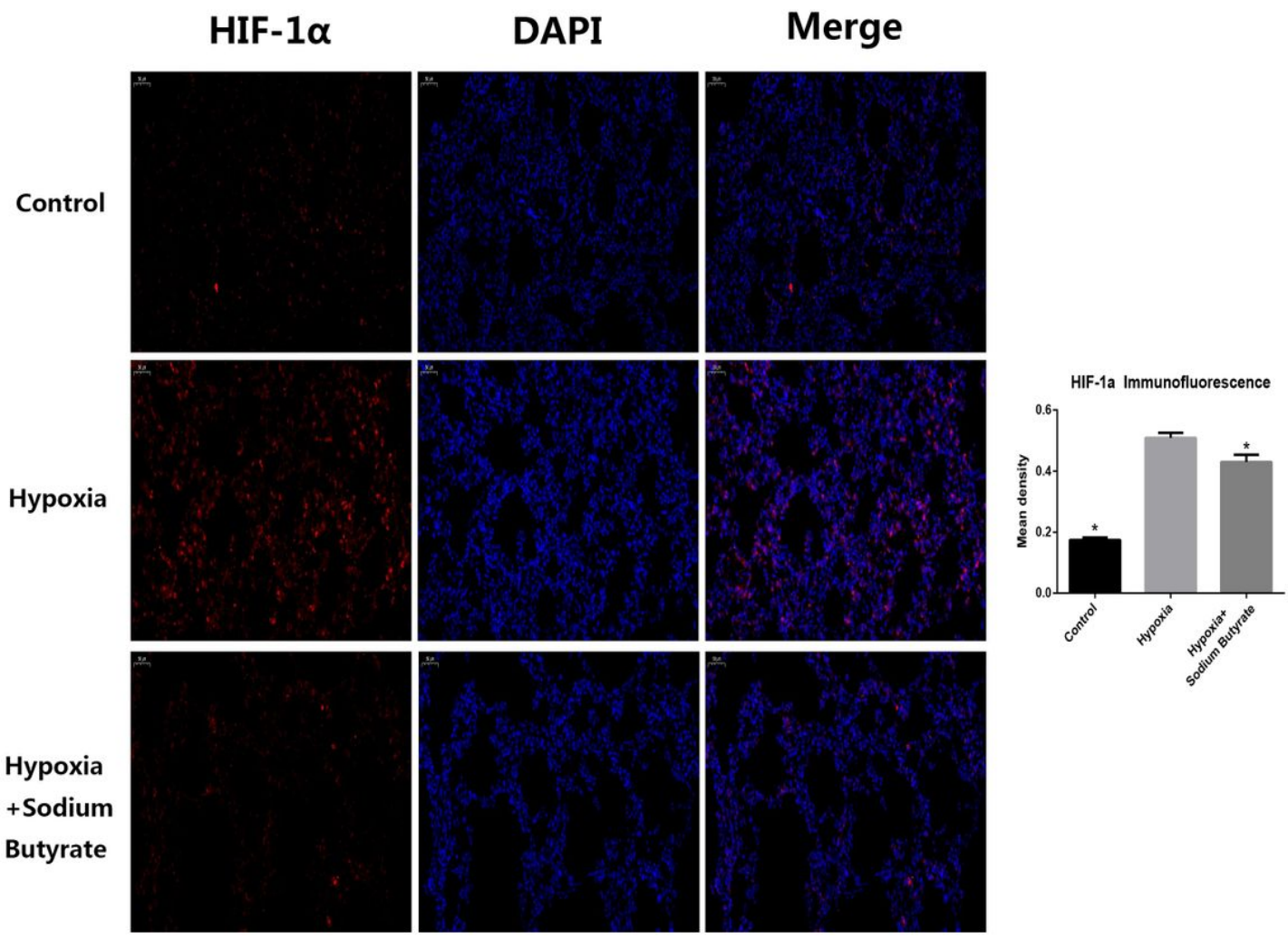

Figure 5

Immunofluorescence of HIF-1α (red) in rats' lungs. Nuclei were counterstained with DAPI (blue). Compared with hypoxia + sodium butyrate and control group, the expression of HIF-1α (red) in hypoxia group increased significantly. Mean density = IOD sum/AREA sum. * $p<0.05$ compared with Hypoxia. (All images are magnified at ×20)

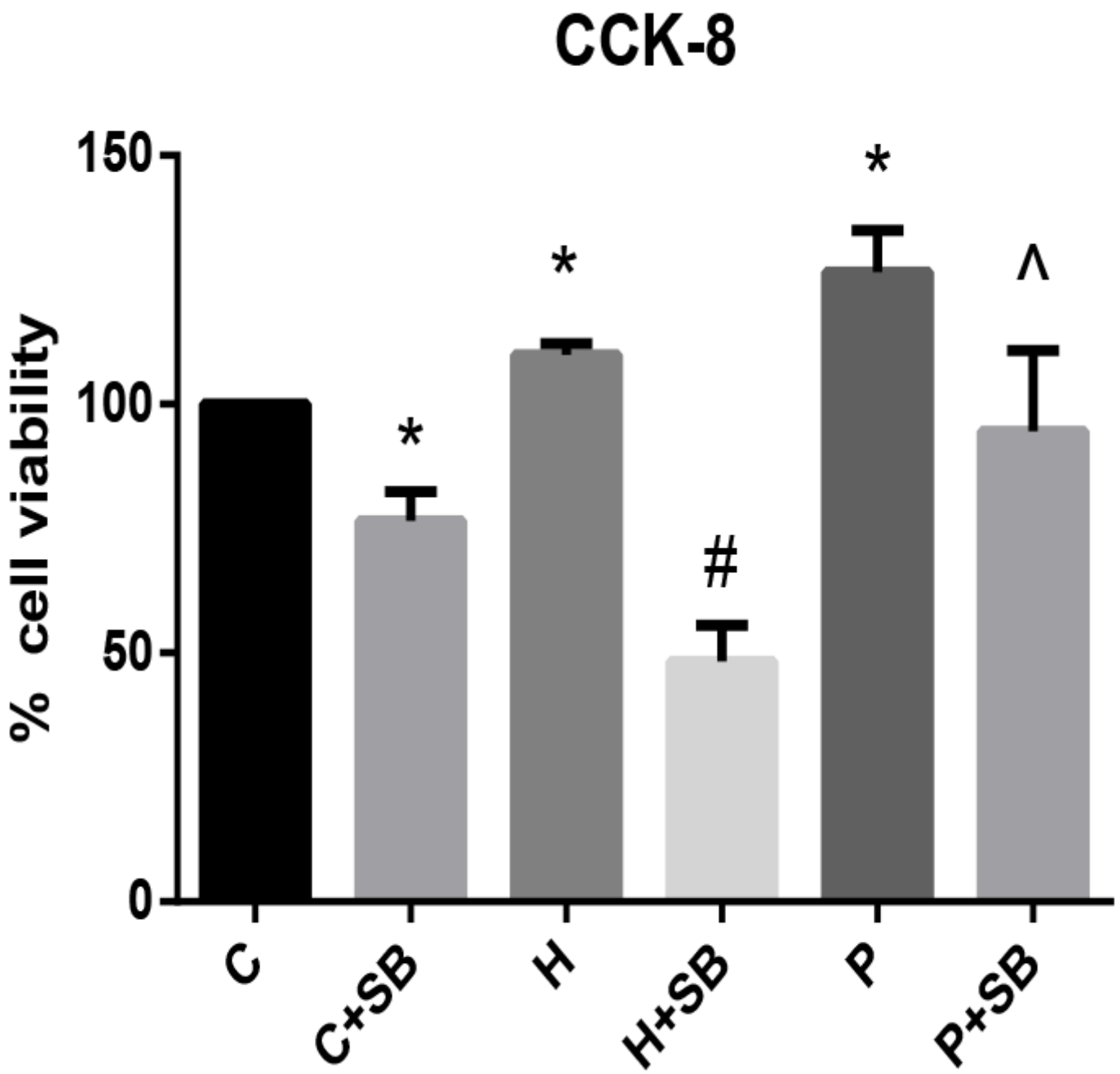

Figure 6

CCK-8 of PASMC (48 hours). cell viability = [A450(sample)-A450(blank)] /[A450(C)-A450(blank)]*100%. C: Control, H: Hypoxia (1%O2), P: PDGF (20 ng/ml), SB: Sodium Butyrate (3 mmol/l). The data are presented as mean±SD. * $p<0.05$ compared with Control, # $p<0.05$ compared with Hypoxia, ^ $p<0.05$ compared with PDGF.

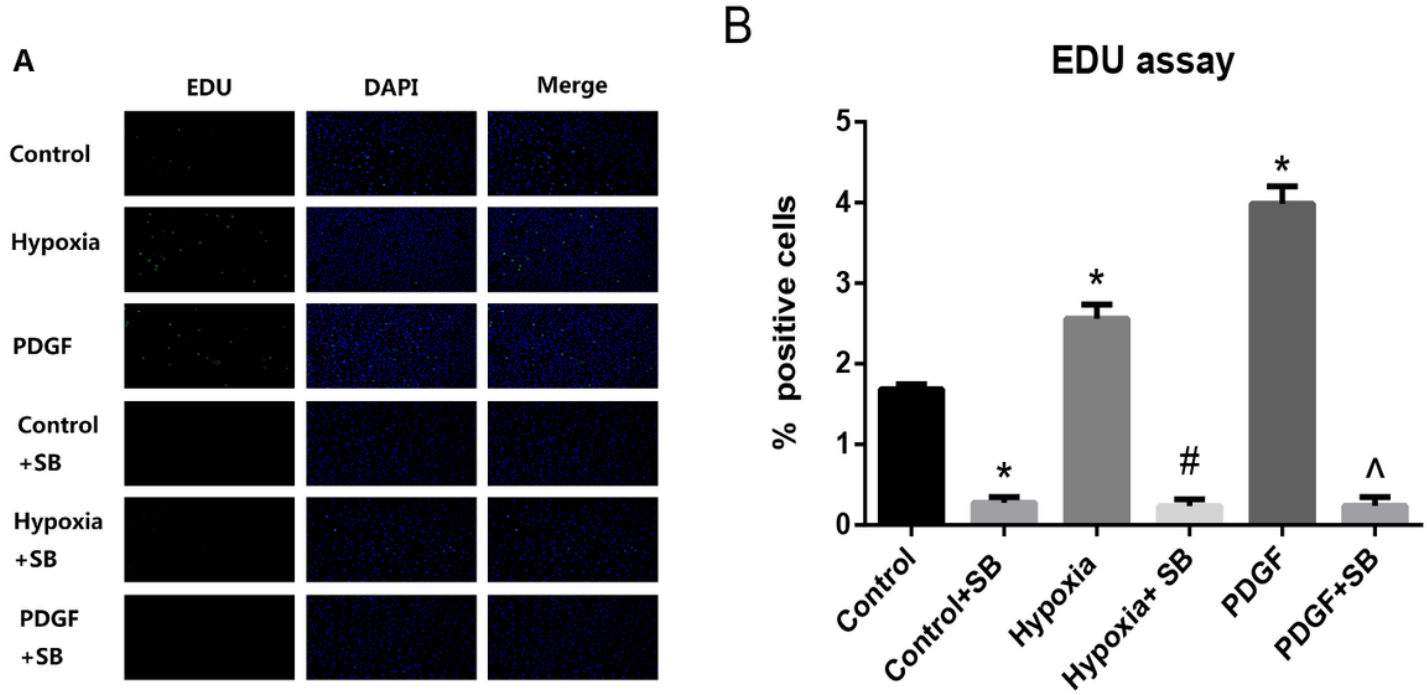

Figure 7

A. EDU (green) assay of PASMC (48 hours). Nuclei were counterstained with DAPI (blue). PDGF (20 ng/ml): Platelet Derived Growth Factor. SB (3 mmol/l): Sodium Butyrate. Hypoxia (1%O2). (All images are magnified at ×100). B. Quantification of EDU assay: results are expressed as percentage of positive cells normalized to the number of nuclei. The percentages of positive cells are presented as mean±SD. * p<0.05 compared with Control, # p<0.05 compared with Hypoxia, ^ p<0.05 compared with PDGF.

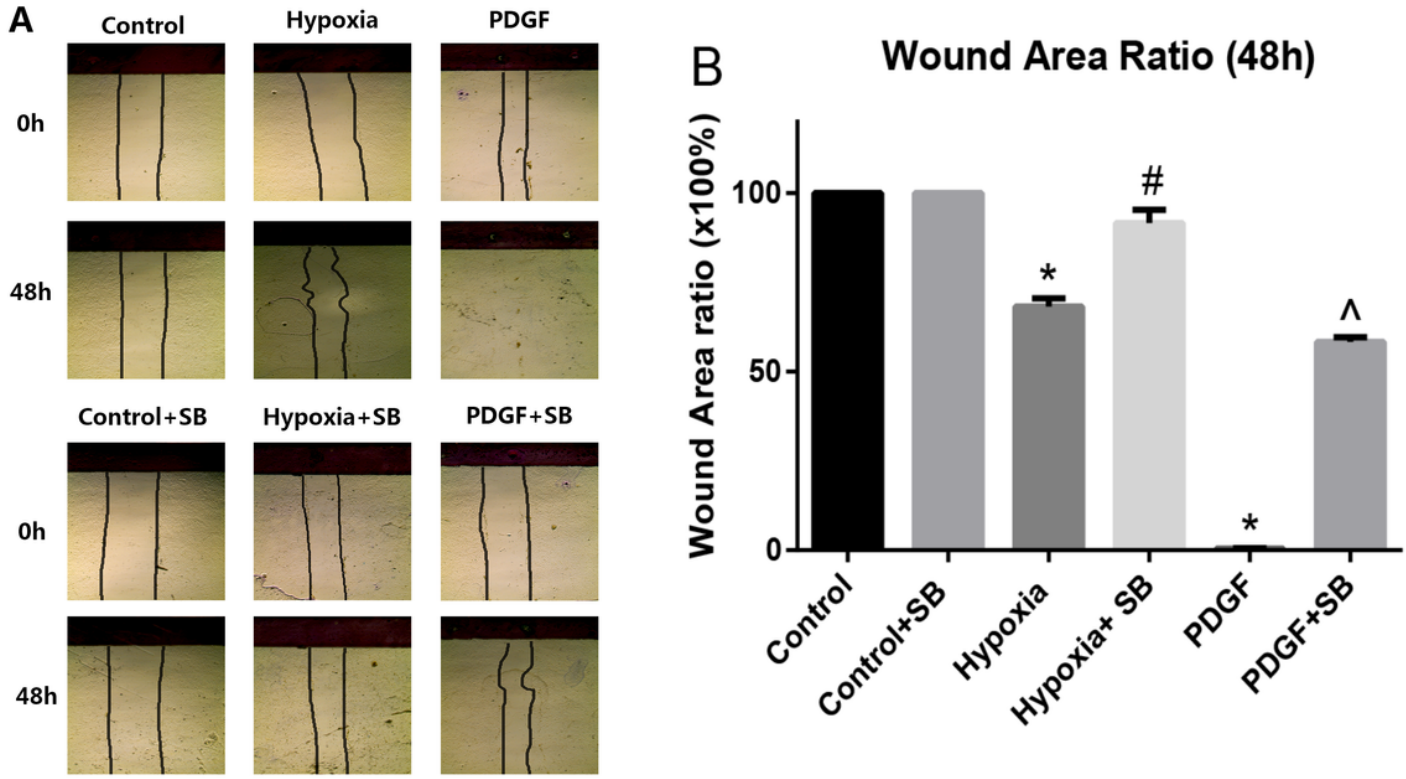

Figure 8

A. Cell migration of PASMC (48 hours). PDGF (20 ng/ml): Platelet Derived Growth Factor. SB (3 mmol/l): Sodium Butyrate. Hypoxia (1%O2). (All images are magnified at ×40). B. Wound area ratio = Wound area (48h) / Wound area (0h) * 100%. The wound area ratios are presented as mean±SD. * p<0.05 compared with Control, # p<0.05 compared with Hypoxia, ^ p<0.05 compared with PDGF.

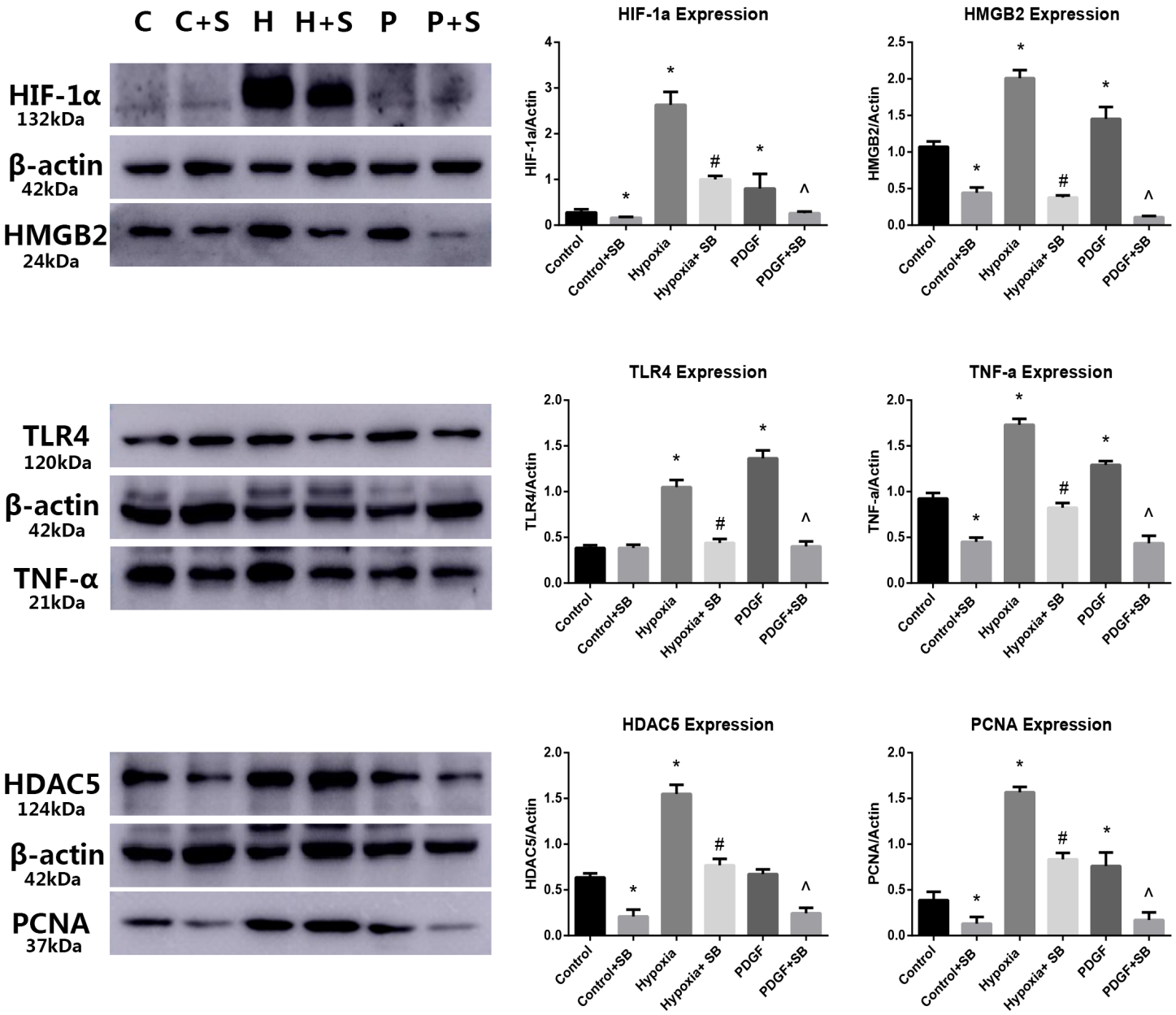

Figure 9

Western blotting of PASMC. C: Control, S/SB: Sodium Butyrate (3 mmol/l), H: Hypoxia (1%O2), P/PDGF: Platelet Derived Growth Factor (20 ng/ml). HIF-1α/β-actin/HMGB2 were from the same gel, TLR4/β-

actin/TNF-α were from the same gel, HDAC5/β-actin were from the same gel, while PCNA was from the different gel with same conditions. All images were automatic exposed. The data are presented as mean±SD. * $p<0.05$ compared with Control, # $p<0.05$ compared with Hypoxia, ^ $p<0.05$ compared with PDGF.

Supplementary Files

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