Impact of Resolvin D1 on the Inflammatory Phenotype of Periodontal Ligament Cell Response to Hypoxia

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Abstract

Objective: Periodontal ligament cells (PDLCs) are critical for wound healing and regenerative capacity of periodontal diseases. Within an inflammatory periodontal pocket, a hypoxic environment can aggravate periodontal inflammation, where PDLCs response to the inflammation would change. Resolvin D1 (RvD1) is an endogenous lipid mediator, which can impact intracellular inflammatory pathways of periodontal/oral cells and periodontal regeneration. It is not clear how hypoxia and RvD1 impact the inflammatory responses of pro-inflammatory PDLCs phenotype. Therefore, this study aimed to test hypoxia could induce changes in pro-inflammatory phenotype of PDLCs and RvD1 could reverse it.

Methods: Human PDLCs were cultured from periodontal tissues from eight healthy individuals and were characterized by immunofluorescence staining of vimentin and cytokeratin. Cell viability was examined by Methyl-thiazolyl-tetrazolium (MTT) assay. To examine the effects of hypoxia and RvD1 on the inflammatory responses of pro-inflammatory PDLCs phenotype, protein levels and gene expressions of inflammatory cytokines and signal transduction molecules were measured by enzyme-linked immunosorbent assay (ELISA), western blotting (WB), and real-time quantitative reverse transcription PCR (real-time qRT-PCR). Alizarin red S staining and real-time qRT-PCR were employed to study the effects of hypoxia and RvD1 on the osteogenic differentiation of pro-inflammatory PDLCs phenotype.

Results: It was found that hypoxia increases the expression of inflammatory factors at

the gene level ($p<0.05$). RvD1 reduced the expression of IL-1β ($p<0.05$) in PDLCs under hypoxia both at the protein and RNA levels. There were increases in the expression of p38 mitogen-activated protein kinase (p38 MAPK, $p<0.01$) and protein kinase B (Akt, $p<0.05$) in response to RvD1. Also, a significantly higher density of calcified nodules was observed after treatment with RvD1 for 21 days under hypoxia.

Conclusion: Our results indicate that hypoxia up-regulated the inflammatory level of PDLCs. RvD1 can reduce under-hypoxia-induced pro-inflammatory cytokines in inflammatory phenotype of PDLCs. Moreover, RvD1 promotes the calcium nodules in PDLCs, possibly by affecting the p38 MAPK signaling pathway through Akt and HIF-1α.

Keywords: Periodontal ligament cells, Resolvin D1, Pro-inflammatory phenotype, Hypoxia, Osteogenesis

Introduction

Periodontitis is caused by a bacterial insult to the periodontal tissues (1), and the primary mechanism is unresolved inflammatory responses (2), which lead to the increased production of inflammatory cytokines and resorption of alveolar bone (3, 4). Within the gingivitis-affected sulcus or periodontal pocket, the resident anaerobic bacteria interact with the host inflammatory reactions resulting in a lower oxygen content or a hypoxic environment (5). Hypoxia is a significant risk factor for the

periodontal disease (6-10) and both intensity and duration of hypoxia is important, where acute exposure of cells to severe hypoxia ($1\%O_2$) can enhance inflammatory responses, accelerate disease progression and aggravate tissue damage but slight hypoxia (5% O_2) have no effect on cell growth even if it lasted 72 hours (7, 11-14). Furthermore, hypoxia-inducible factor-1 (HIF-1) is a critical player in hypoxia-mediated tissue responses for activating nuclear factor-kappa B (NF-κB) in PDLCs (15, 16).

Specialized mediators of inflammation (SPMIs) are specific lipid molecules that directly contribute to the resolution of inflammation and simultaneously enhance wound healing (17). RvD1 is potent endogenous SPMIs derived from docosahexaenoic acid (DHA) and has a resolving effect, which has been demonstrated in many different areas (18-23). However, the pro-resolution and pro-regenerative effects of RvD1 on periodontium are not well known.

It has been reported that RvD1 regulates inflammation (24) through PI3K-Akt signaling pathway on the surface of PDLCs (25, 26). The Akt signaling pathway can promote hypoxia-induced apoptosis of PDLCs (8) and hypoxic-condition-induced expression of HIF-1α and IL-1β (11), where IL-1β can affect HIF-1α expression through PI3K-Akt-mTOR axis and NF-κB signaling pathway (16). Osteogenetic properties of PDLCs can be stimulated by HIF-1 and PI3K-Akt pathway (27, 28) and HIF-1 promotes osteogenic differentiation of PDLCs (6, 7, 29, 30). Furthermore, it has been reported that osteogenic differentiation of PDLCs under hypoxic condition was mediated by the p38 MAPK signaling pathway (31). Despite these progresses, it

is not clear how RvD1 could impact the cellular responses in PDLCs under hypoxia.

Therefore, in the present study we aimed to test the role of hypoxia in the impact of pro-inflammatory PDLCs phenotype and whether RvD1 could reverse hypoxia-induced changes in PDLCs, and then report the possible mechanisms in place during such a process.

Materials and Methods

Isolation and culturing of PDLCs

The study was approved by Ethics Committee of School of Stomatology in Lanzhou University (approval number: LZUKQ-2020-26). Human PDLCs were isolated from periodontal biopsies obtained from eight healthy individuals with no clinical signs of periodontal disease. Cells were isolated from middle 1/3 of the root of isolated teeth as described before (17) and were divided into three cell lines. Briefly, periodontal tissue was obtained and rinsed with phosphate-buffered saline (PBS) containing penicillin (1000 U/mL) and streptomycin (1000 mg/mL) for 3-4 times. Minced pieces of periodontal tissues were placed into 25 cm^2 tissue culture flask (Beaver, Guangzhou, China) and were cultured with minimal essential medium alpha modification (α-MEM, Hyclone™, Pittsburgh, USA) containing 10% fetal bovine serum (FBS, Abwbio, Shanghai, China) with 1% penicillin (100 U/mL) and streptomycin (100 mg/mL) at 37℃ in a humidified environment containing 5% CO_2. Outgrowing PDLCs were routinely passaged using 0.025% trypsin. The medium was replaced every three days with a fresh one. Morphology of PDLCs was observed

under an inverted microscope (OLYMPUS, CKX53, Tokyo, Japan).

Identification of PDLCs

PDLCs were seeded into 6-well plates at a density of 7×10^4 cells per well. Cells were washed two times with PBS and then were fixed with 4% formalin solution for 30 min. Vimentin (00066574, Proteintech™, Wuhan, China) was used as a fibroblastic marker at a dilution of 1:200 in universal antibody diluent (WB100D, NCM Biotech, Suzhou, China). Keratin (ZM-0069, ZSGB-BIO, Beijing, China) as a negative marker was applied to the identification of epithelial cells. Hoechst 33342 (Solarbio®, Beijing, China) was used to label the nuclei. The images were captured under a fluorescence microscope (OLYMPUS, CKX53, Tokyo, Japan).

Alizarin red S staining

The osteogenic potential of cells was measured by Alizarin Red S staining (AR-S, Cat#G1452, Solarbio®, Beijing, China). Cells were seeded in 6-well plates at 5×10^4 cells per well and were incubated in the osteogenic induction medium (OIM, Stemcell™, Ottawa, Canada). The culture medium was refreshed every three days. After 21 days of osteogenic differentiation, the medium was removed. After fixation, cells were stained with Alizarin Red S for 5 min and then were washed three times with PBS. Images were taken by an inverted microscope (OLYMPUS, CKX53, Tokyo, Japan). ImageJ was used to count the positive staining of calcium nodules with a threshold of 0-60 in eight fields. The area fraction of the positive staining to the total area was calculated. After Alizarin Red S staining images were taken, 10%

cetylpyridinium chloride (yuanyeBio™, Shanghai, China) was used to dissolve the mineralized nodules for quantitative analysis. The absorbance was measured at 560 nm.

Cell proliferation test by MTT

Cell proliferation was measured by 3-[4, 5- dimethylthiazol-2- yl]-2, 5-diphenyltetrazolium bromide (MTT, Solarbio®, Beijing, China) assay. PDLCs were seeded in 96-well plates with 5000 cells per well and were cultured for 24 h. Cells were treated with lipopolysaccharide (LPS, Solarbio®, Beijing, China) at different doses of 0.01, 0.1, 1, 10 μg/ml for 72 h. After this treatment, 20 μl MTT was added to each well. The cells were incubated for another four hours with 5% CO_2 at 37 ℃. After adding 150 μl dimethyl sulfoxide (DMSO) to dissolve the formed purple formazan dye, the absorbance at 490 nm was measured by Infinite 200^{Pro} microplate reader (Tecan®, Austria).

Induction of LPS and hypoxia

Lipopolysaccharide (LPS) was used to establish an pro-inflammatory phenotype for PDLCs. PDLCs were seeded into 6-well plates at a density of 7×10^4 cells per well and were incubated with 0.01, 0.1 or 1 μg/mL LPS for 3 days. An enzyme-linked immunosorbent assay (ELISA) was used to measure the level of IL-1β (Jianglai, Shanghai, China).

Based on the results of MTT and ELISA tests, LPS treatment with a dose of 0.1 μg/ml was found to show no toxicity on cell proliferation and can significantly stimulate

IL-1β expression, therefore it was chosen in the later experiments. PDLCs were seeded into 6-well plates at a density of 7×10^4 cells per well under three different conditions (Control, LPS, LPS+RvD1) under normoxic or hypoxic conditions. Resolvin D1 (RvD1, Cayman Chemical, Ann Arbor, Michigan) at 100 ng/ml was used according to previous studies (17, 32). The normoxic groups were cultured in an incubator with 5% CO_2 at 37 ℃. The hypoxic groups were placed in the hypoxic workstation (Maworde, Jiangsu, China) in which the pressure of oxygen (pO_2) is 7.14 mmHg [1.0% (vol/vol) O_2], the pressure of carbon dioxide is 35 mmHg [5% (vol/vol) CO_2] and the temperature is 37 ℃. After 3 days culturing, cells and supernatants were collected separately for different measurements.

Inflammatory cytokines secreted by PDLCs

The supernatants of different groups were collected and the levels of inflammatory cytokines were tested using human IL-1β enzyme-linked immunosorbent assay (ELISA) kit (Jianglai, Shanghai, China), human TNF-α ELISA kit (Jianglai, Shanghai, China) and human IL-6 ELISA kit (Neobioscience, Shenzhen, China) according to manufacturers' protocols. The absorbance at 450 nm was measured by Infinite 200Pro microplate reader (Tecan®, Austria). All the experiments were performed in triplicate.

Protein isolation and western blot analysis

Total protein was extracted with radio immunoprecipitation assay buffer (RIPA buffer, Solarbio®, Beijing, China) supplemented with phenylmethanesulfonyl fluoride (PMSF, Solarbio®, Beijing, China) and phosphatase inhibitors (AbMole, American).

Electrophoresis was performed with 10% separation gel to separate the protein components. After the transferring, the polyvinylidene fluoride (PVDF, Amershom^{TM}, Germany) membrane was incubated in primary antibodies overnight. The primary antibodies were HIF-1α, nuclear factor-kappa B (NF-κB), p38 mitogen-activated protein kinase (p38 MAPK), protein kinase B (Akt), runx family transcription factor 2 (Runx2), osteopontin (OPN) and β-actin. All the primary and secondary antibodies were purchased from ProteinTech, Wuhan, China. Protein bands were imaged using a chemiluminescence apparatus (VILBER, France) with NcmECL Ultra (NCM Biotech, Suzhou, China). Imaging data was analyzed by ImageJ.

Real-time quantitative reverse transcription PCR (real-time qRT-PCR)

Total RNA was extracted with TRIzol (Ambion®, Austin, USA) protocol. EasyQuick RT MasterMix (Cwbio, Beijing, China) was used to perform reverse transcription. Next, real-time qRT-PCR was performed using UltraSYBR Mixture (Cwbio, Beijing, China) on Rotor-Gene real-time PCR cycler (QIAGEN, Frankfurt, Germany). Beta-actin was used as a reference gene. The primer sequences are shown in Table 1. Data were analyzed by the comparative ($2^{-\triangle\triangle CT}$) method.

Statistical analysis

In the present study, each experiment was repeated at least three times. GraphPad Prism (GraphPad Software, USA) was employed for data analysis and two-way ANOVA was used to find the differences between the groups. A *p*-value less than 0.05 ($p<0.05$) was considered statistically significant.

Results

Culture and identification of PDLCs

PDLCs were found to show a typical fibroblastic morphology (Figure 1a). The cells were stained positively for vimentin and negatively for cytokeratin (Figure 1b). After 21 days of osteogenetic induction, the positive alizarin red S staining of mineralized nodules was observed (Figure 1c).

Cell proliferation and secretion of IL-1β in PDLCs treated with LPS

For the four groups on day 3, at doses of 0.01 and 0.1 μg/ml, LPS had no effect on the proliferation of PDLCs (Figure 1d). PDLCs treated with 0.1 μg/mL LPS could significantly increase the level of IL-1β (Figure 1e). Therefore, LPS at the dose of 0.1 μg/ml was chosen for subsequent experiments.

Hypoxia promoted inflammatory responses of PDLCs

Interestingly, the effects of LPS on inflammatory responses were enhanced under the hypoxia condition. Under hypoxia, the expression of IL-1β, IL-6 and TNF-α were significantly higher in all three groups compared with normoxia at the gene level (Figure 2 d-f). Also, the detections of HIF-1α and p38 were significantly increased under hypoxia compared with normoxia (Figure 3), where Akt was increased in the absence of LPS.

RvD1 reduced the inflammatory responses of LPS-stimulated PDLCs induced by hypoxia

Under both normoxic and hypoxic conditions, LPS could induce an inflammatory

profiling in PDLCs, where the RvD1+LPS group showed a significantly lower level of inflammatory response compared with the LPS group (Figure 2a, d-f). Under normoxia, RvD1 significantly reduced the level of IL-1β. Under hypoxia, RvD1 significantly reduced the expressions of IL-1β, IL-6 and TNF-α at the gene level. In the cellular signaling, RvD1+LPS stimulated a higher level of Akt and p38 compared with LPS (Figure 3).

Hypoxia promoted osteogenic differentiation of PDLCs

Alizarin red S staining showed a higher cell density in normoxia than in hypoxia, but the formation of calcium nodules was significantly promoted under hypoxia (Figure 4). The RvD1+LPS group had a higher level of calcium nodules than the LPS group under the hypoxic condition. Hypoxia improved the protein expressions of OPN and Runx2 in RvD1+LPS group compared with the control (Figure 3). However, the osteogenic genes alkaline phosphatase (ALP) and OPN were down-regulated under hypoxia. There were up-regulated expressions of ALP and OPN in the LPS and RvD1+LPS groups compared with the control (Figure 5). Under normoxia, the expression of Runx2 was down-regulated to a larger extent in the RvD1+LPS group compared with the LPS group.

Discussion

Periodontitis leads to tissue damage and resorption of alveolar bone and PDLCs play a major role in promoting wound healing. In the present study, the impacts of the LPS-stimulated inflammatory response of PDLCs under normoxic and hypoxic

conditions were investigated and evidence was provided on the anti-inflammatory effect of RvD1 on PDLCs under hypoxia.

PDLCs are a group of cells, which include fibroblasts, osteoblasts, epithelial rests of Malassez, and cementoblasts (33, 34). The results of the characterization of PDLCs by vimentin staining and the examination of osteogenic differentiation by alizarin red S staining after 3 weeks of induction were in line with those of previous studies (35, 36). LPS, which was used as a component of the outer membrane of Gram-negative bacteria, is an important virulence factor of *P. gingivalis* activating the immune system via transmembrane Toll-like receptors (TLRs) (37). It was found that PDLCs acquired a pro-inflammatory phenotype after being treated with LPS (Figure 1e), which mimics a local microenvironment at the periodontitis site. LPS groups were used to make comparisons with LPS+RvD1 groups to test whether RvD1 could reverse hypoxia-induced changes in PDLCs.

It has been reported that the severity of periodontitis is positively correlated with hypoxia (12). A hypoxic gradient exists in deep periodontal pockets due to Gram-negative anaerobic pathogens, the inflammatory infiltrate and the impaired microcirculatory perfusion (38). The local hypoxic microenvironment is considered a consequence of wound healing, development, smoking habits, and oral inflammation (39). PDLCs can also gain a pro-inflammatory phenotype when exposed to hypoxia. Since the amount of oxygen in wounds and infected tissue is usually 1-2% (40), an oxygen concentration of 1% was selected to simulate hypoxia in the present study. A hypoxic environment can regulate the effects of LPS, such as the expression of IL-1

and TNF-α (9, 10). Under the stimulation of hypoxia, the level of inflammatory cytokines in PDLCs increased (Figure 2), which is similar to the findings of a previous study (41). The expression of HIF-1α, Akt, and p38 was also increased under the hypoxic condition (Figure 3), indicating that hypoxia may affect the inflammatory response through Akt and p38 signaling pathways. It is of note that this phenomenon could be reversed by RvD1.

Upon application of RvD1, the expression of IL-1β and TNF-α at the gene level was decreased in a hypoxic environment (Figure 2). Since hypoxia generated a pro-inflammatory phenotype of PDLCs, the anti-inflammatory effect of RvD1 was more noticeable, thus indicating that RvD1 could reverse the effect of hypoxia. NF-κB and p38 MAPK signaling pathways are the most common inflammatory regulatory pathways (11, 42). With respect to reversing the pro-inflammatory phenotype of PDLCs obtained under hypoxia and in the presence of LPS, RvD1 was found to have no effect on the expression of NF-κB by Akt (Figure 3). Recchiuti et al. showed that the resolving effect could also be influenced by miR-146b (43), which may explain these findings by suggesting that RvD1 presumably induce its anti-inflammatory effect through microRNA. Furthermore, the detection of phosphorylated NF-κB was to be expected. The expression of p38 was up-regulated in a hypoxic environment (Figure 3), which might be another reason for the non-decrease characteristic of NF-κB expression in a hypoxic environment.

Osteogenic differentiation of PDLCs was promoted by hypoxia. Although a prolonged hypoxic environment inhibited cell proliferation, the relative calcium deposition was

significantly increased in the pro-inflammatory phenotype of PDLCs with RvD1 (Figure 4). Real-time qRT-PCR results showed that hypoxia reduced the expression of OPN in LPS-stimulated cells (Figure 5), which may suggest that hypoxia induces a reduction in osteoclast formation and resorptive activity. Nevertheless, the protein level of OPN (Figure 3) was different from that of the gene (Figure 5), indicating that early hypoxia promoted osteoclast. Moreover, in the RvD1+LPS group OPN was down-regulated compared with the LPS group (Figure 3), suggesting that RvD1 inhibits bone resorption. However, the RNA level of ALP was decreased under three days of hypoxia compared with normoxia, indicating that osteoblast formation was reduced. One possible reason could be that hypoxia resulted in a lower density of PDLCs, thus inducing a lower degree of osteoblast differentiation on day 3. Hence, in the present study, the positive effect of RvD1 on the osteogenesis may not be identified at the mRNA level.

Akt and p38 signaling pathways could promote osteoblast proliferation and regulate osteogenic markers (44, 45). Akt is the crucial stimulus for osteogenesis (27, 28, 46, 47). Also, maxillofacial bone regeneration could be promoted via the PTEN/PI3K/Akt/HIF-1α pathway. In the present study, the protein level of Akt was found to be improved after treatment by RvD1 (Figure 3). Previous studies have shown that the osteogenic differentiation of human bone marrow mesenchymal stem cells and human periodontal ligament stem cells could be promoted by activating the p38 signaling pathway under hypoxia (31, 48-50). In the present study, both HIF-1α and p38 were found to be increased under hypoxia (Figure 3), suggesting that the

osteogenic effect of RvD1 was mediated through Akt, HIF-1α and p38.

RvD1 reverses the pro-inflammatory phenotype of PDLCs while increasing the impact of hypoxia on their osteogenetic function. Thus, the results might indicate that RvD1 could act as two different and independent mediators for PDLCs under two different microenvironments. One possible anti-inflammatory pathway Akt/HIF-1α/NF-κB of RvD1 was still to be studied and one probable pro-regenerative pathway Akt/HIF-1α/p38 of RvD1 was verified. Further studies are required to confirm the signaling pathways of RvD1 under hypoxia.

Conclusion

Hypoxia can promote the cellular response and osteogenic differentiation of PDLCs. Meanwhile, RvD1 can mediate the hypoxia-induced changes in pro-inflammatory PDLCs phenotype. RvD1 can reduce pro-inflammatory cytokines in LPS-stimulated cells induced by hypoxia. Also, RvD1 promotes the calcium nodules in PDLCs, possibly by affecting the p38 MAPK signaling pathway through Akt and HIF-1α.

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Contributions of the authors

Twelve authors contributed to this article, namely J.C., J.L., J.Y., X.L., X.W., S.L., K.M., H.W., Y.X., M.M., A.K. and Z.X. The contributions of each one of them are as follows. J.C.: cell experiment, data analysis and writing. J.L., J.Y., X.L., X.W. and S.L.: data analysis and writing. K.M.: conception and revision. H.W.: correspondence and conception. Y.X.: conception. M.M.: conception and revision. A.K.: revision and conception. Z.X.: supervision, design and revision of the manuscript. All authors have read and agreed to the submitted version of the manuscript.

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Table

Table 1: Primer sequences used for real-time qRT-PCR. F, Forward; R, Reverse.

Gene	Primer sequence (5′-3′)
TNF-α	F: AGTAACATGGAGCTGCAGAGGATGA
	R: TGGAGACAGGGACATCAGTCG
IL-6	F: CCCCTGACCCAACCACAAAT
	R: GTGCCCATGCTACATTTGCC
IL-1β	F: GTGTGTGGAGAGCGTCAACC
	R: ACAGTTCCACAAAGGCATCCCAG
ALP	F: ACAGCCGCCAAGAACCTCA
	R: CACTGTCTGGCACATGTTTGTCTAC
Runx2	F: CACTGGCGCTGCAACAAGA
	R: CATTCCGGAGCTCAGCAGAATAA
β-actin	F: GAAACTACCTTCAACTCCATC
	R: CTAGAAGCATTTGCGGTGGAC
OPN	F: CAGTTGTCCCCACAGTAGACAC
	R: GTGATGTCCTCGTCTGTAGCATC

Figure Legends

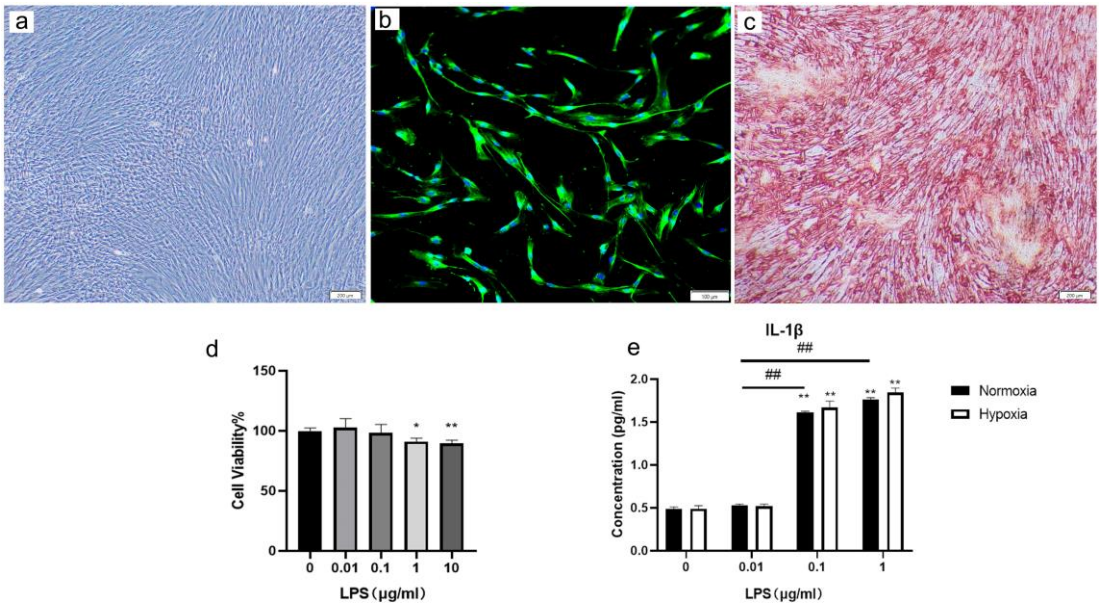

Figure 1. Identification and osteogenic differentiation of PDLCs. a: Morphology of PDLCs at passage 1: cells exhibited morphologies with triangular, spindle-like, cuboid, and short fusiform shapes. The scale bar is 200 μm. b: PDLCs were positive for vimentin (green) and cell nuclei stained with Hoechst (blue), where cytokeratin was not detected. The scale bar is 100 μm. c: Positive staining of Alizarin red S in PDLCs after 21 days culturing with osteogenic inducing medium. The scale bar is 200 μm. d: Cell viability test by MTT after 3 days of treatment with LPS. LPS at doses of 0.01 and 0.1 μg/ml had no effect on cell viability. e: IL-1β secretion by PDLCs was measured by ELISA kit. LPS treatments at 0.1 and 1 μg/ml doses significantly increased the level of IL-1β under normoxic and hypoxic conditions, where there was no significant difference between 0.1 and 1 μg/ml groups. All the experiments were performed in triplicate. The data are presented as means ± SD (*$p<0.05$, **$p<0.01$).

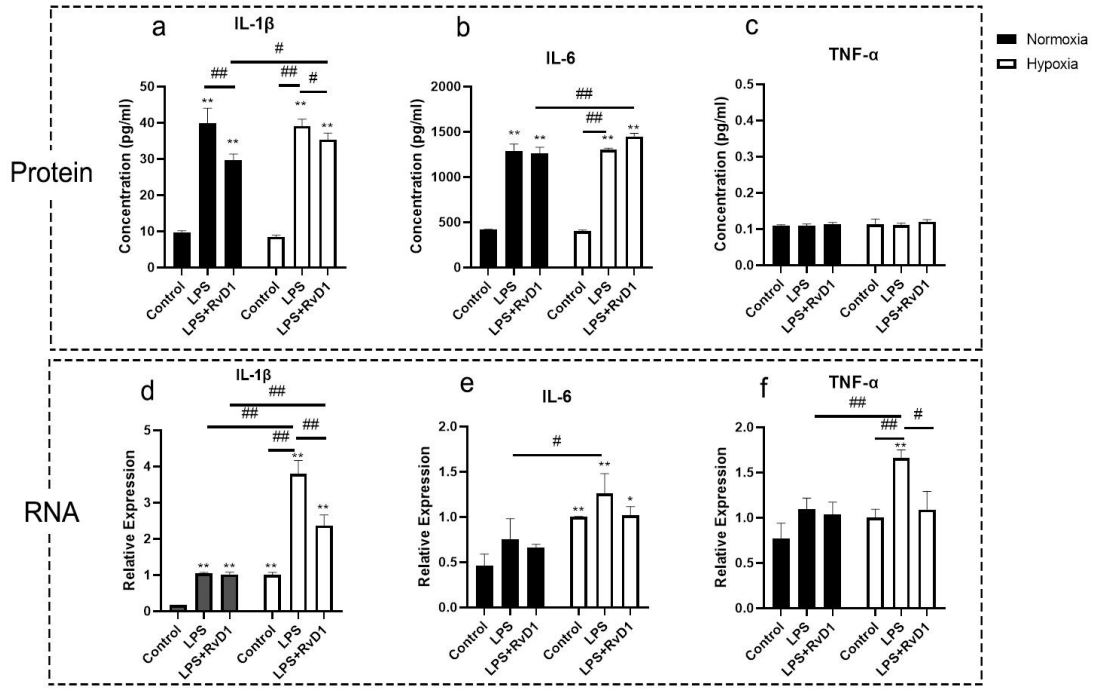

Figure 2. Impact of hypoxia and RvD1 on the inflammatory cytokines. a-c represent the level of cytokine production measured by ELISA. The levels of TNF-α were not detectable, because they were below the detection range. d-f represent the level of mRNA expression measured by real-time qRT-PCR. Under hypoxia, the expression of the inflammatory cytokines was enhanced by LPS stimulation. Both IL-1β and TNF-α expressions were decreased in LPS+RvD1 group under the hypoxic environment. All the experiments were performed in triplicate. The data are presented as means ± SD (*$p<0.05$ vs. control, **$p<0.01$ vs. control, #$p<0.05$, ##$p<0.01$).

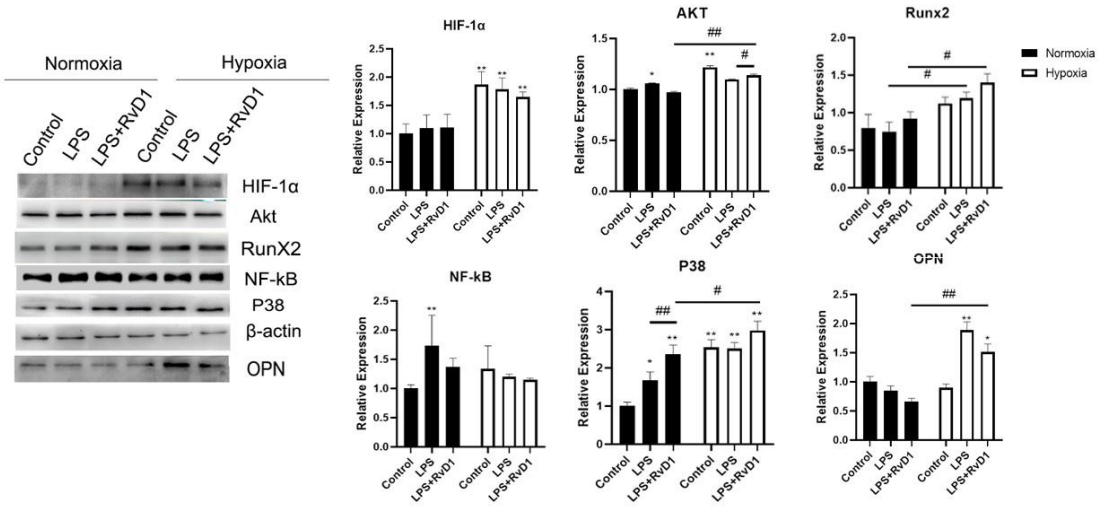

Figure 3. The effect of hypoxia and RvD1 on cell signaling and osteogenic markers HIF-1α, NF-κB, p38, Akt, Runx2, and OPN in PDLCs. Hypoxia increased the expression of HIF-1α, p38 and Akt. Under hypoxic conditions, RvD1 could increase the expression of p38 and Akt, but decreased the expression of OPN in LPS-stimulated cells. All the experiments were performed in triplicate. The data are presented as means ± SD (*$p<0.05$ *vs. control*, **$p<0.01$ *vs. control*, #$p<0.05$, ##$p<0.01$).

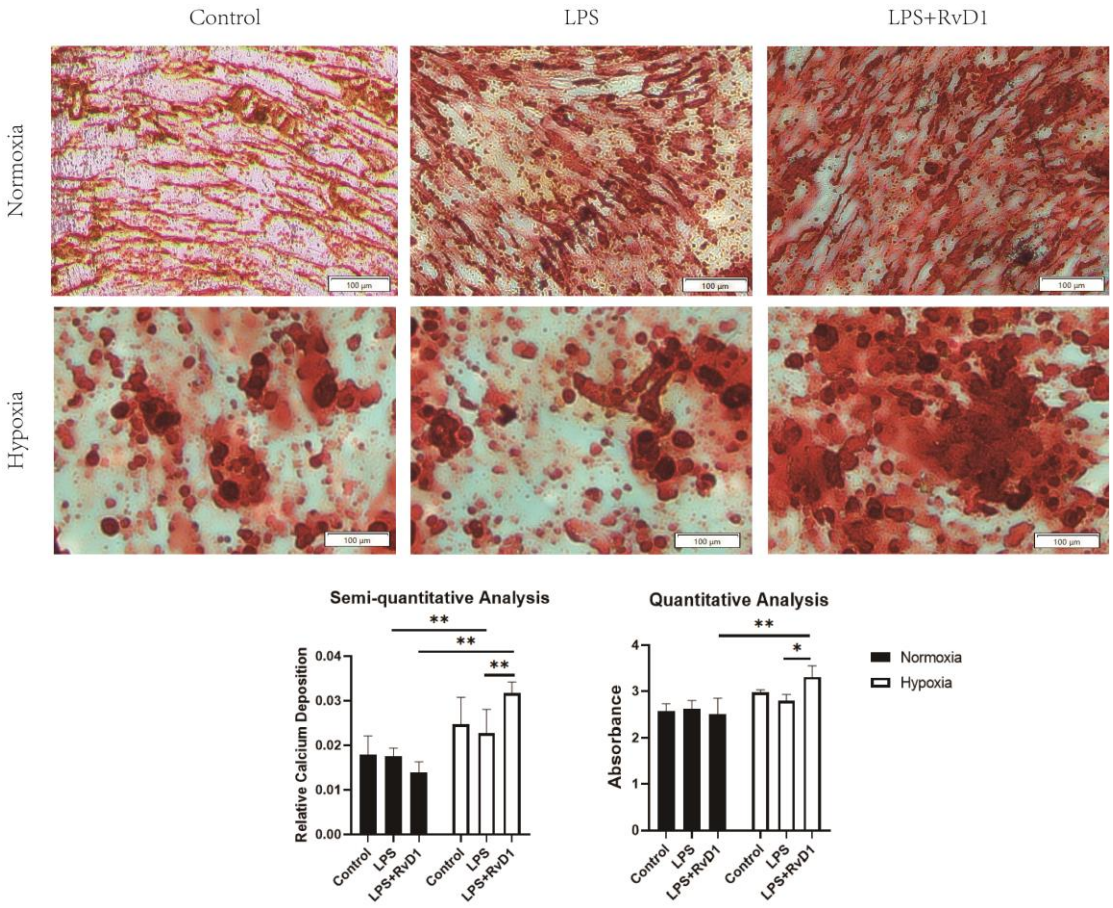

Figure 4. Alizarin red S staining of PDLCs after 21 days of osteogenesis inducing medium culture. After the staining was dissolved in 10% cetylpyridinium chloride, a quantitative measurement was performed. The semi-quantitative analysis was the ratio of the amount to the total area of normalized calcium nodules. Cell density decreased under hypoxia, but the calcium nodules were not decreased and RvD1 promoted their formation. The scale bar is 100 μm. All the experiments were performed in triplicate. The data are presented as means ± SD (*$p<0.05$, **$p<0.01$).

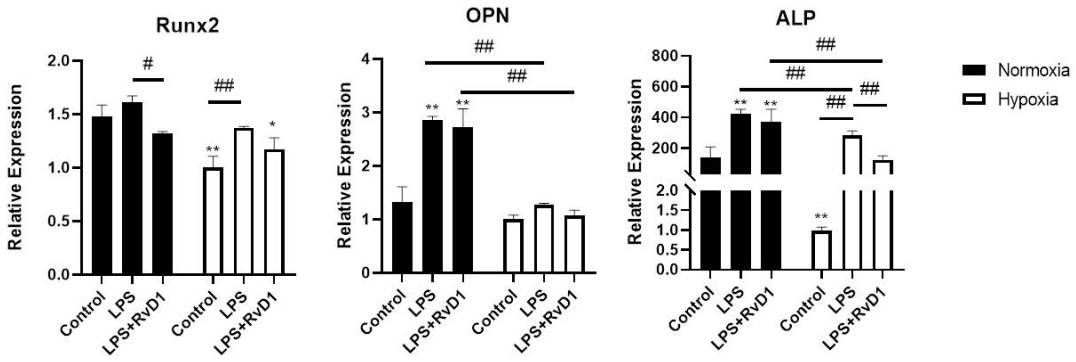

Figure 5. The effect of hypoxia and RvD1 on the mRNA expression of ALP, OPN and Runx2 in PDLCs. Under a hypoxic condition, LPS promoted the expression of ALP and Runx2. Compared with normoxia, the expression of ALP and OPN were decreased after treatment with RvD1 under hypoxia. All the experiments were performed in triplicate. The data are presented as means ± SD (**p<0.05 vs. control, **p<0.01 vs. control, #p<0.05, ##p<0.01*).