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Prolyl hydroxylase inhibitors increase the production of vascular endothelial growth factor by periodontal fibroblasts

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Background and Objective: Pharmacological inhibitors of prolyl hydroxylases (PHDs) can induce a proangiogenic response that favors wound healing and bone regeneration. However, the response of periodontal cells to PHD inhibitors is unknown.

Material and Methods: To determine the effects of PHD inhibitors on periodontal cells, we exposed human fibroblasts from the gingiva and the periodontal ligament to dimethyloxallyl glycine, desferrioxamine, L-mimosine and $CoCl_2$. Viability, proliferation, and protein synthesis were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), [^{3}H]thymidine, and [^{3}H]leucine incorporation, respectively. The levels of Ki67, hypoxia-inducible factor 1α (HIF-1α), p27, phosphorylated c-Jun N-terminal kinase (JNK) and phosphorylated p38 were determined by immunohistochemistry and western blotting. Vascular endothelial growth factor (VEGF) mRNA levels were measured by quantitative PCR. Protein levels of VEGF and interleukin (IL)-6 were evaluated by immunoassays.

Results: We found that PHD inhibitors, while leaving cell viability unchanged, reduced proliferation and protein synthesis. This was paralleled by decreased Ki67 levels and increased p27 levels, suggesting that PHD inhibitors provoke growth arrest. Independently from this response, PHD inhibitors stabilized HIF-1α and increased the production of VEGF. This increase of VEGF was observed in the presence of proinflammatory IL-1 and pharmacological inhibitors of JNK and p38 signaling. Moreover, PHD inhibitors did not modulate expression of IL-6 and the phosphorylation of JNK and p38.

Conclusion: These results suggest that PHD inhibitors enhance the production of VEGF in periodontal fibroblasts, even in the presence of proinflammatory IL-1. The data further suggest that PHD inhibitors do not provoke a significant pro-inflammatory or anti-inflammatory response in this *in vitro* setting.

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Angiogenesis is a two-sided sword in periodontology: while angiogenesis is a pathologic factor in the onset and the progression of tissue destruction (1), angiogenesis is essential for the repair and regeneration of damaged tissue (1,2). Inflammation increases vessel formation and permeability, which is controlled by angiogenic factors (3,4). This results in an increased influx of inflammatory cells, thereby enhancing the inflammatory environment (5).

Consequently, angiogenesis is considered an integral part of the inflammatory process. However, angiogenesis is also an essential factor of regeneration, which is impeded when inflammation has ceased. Preclinical models have revealed that decreased angiogenesis is associated with compromised wound healing and bone regeneration (6,7). Therapies that stimulate angiogenesis can support periodontal repair and regeneration – similarly to bone regeneration and wound healing (8,9).

Angiogenesis can be stimulated by the inhibition of oxygen sensors (10). This strategy has been shown to support bone regeneration and wound healing, suggesting that inhibition of oxygen sensors might also be feasible to support periodontal regeneration (11,12). Oxygen tension can be controlled by prolyl hydroxylase domain-containing proteins also known as prolyl hydroxylases (PHDs) (10). PHDs are active under normoxia, causing the degradation of the labile transcription factor hypoxia-inducible factor 1 (HIF-1α) (10). PHDs are inactive under hypoxia, and thus HIF-1α is maintained and can induce the expression of proangiogenic molecules, such as vascular endothelial growth factor (VEGF) (10). Therefore, inhibition of PHDs causes a cellular response that typically occurs under hypoxia (10). PHD inhibitors target the 2-oxoglutarate-dependent oxygenases or complex Fe^{2+} (13). Typical PHD inhibitors are dimethyloxalylglycine (DMOG), desferrioxamine (DFO), L-mimosine (L-MIM) and $CoCl_2$ (13).

Previously it has been shown that PHD inhibitors increase the expression of VEGF in osteoblasts (12), osteoclasts (14), endothelial cells (15) and epithelial cells *in vitro* (15). In addition to the proangiogenic capacity, PHD inhibitors can modulate the inflammatory response (10). Proinflammatory and anti-inflammatory effects have been reported (10,16–18). These include modulation of the production of proinflammatory cytokines and activation of mitogen-activated kinases such as c-Jun N-terminal kinase (JNK) and p38 (16–19). Although *in vitro* data on the response of cells from bone and soft tissue are available, the response of periodontal fibroblasts to PHD inhibitors is still unclear. Thus, studies that investigate the response of fibroblasts from the periodontium under basal conditions and in the presence of proinflammatory cytokines are required.

In the present study we took advantage of our established *in vitro* system utilizing periodontal fibroblasts isolated from the gingiva (GF) and the periodontal ligament (PDLF). We assessed the impact of PHD inhibitors on the production of VEGF by GF and PDLF. To determine possible toxic effects of the PHD inhibitors we measured viability, proliferation and protein synthesis of GF and PDLF. To understand the impact on the response to inflammatory cytokines we assessed interleukin (IL)-1-induced IL-6 production and phosphorylation of the mitogen-activated kinases JNK and p38. This proof-of concept study provides the first insights into the ability of PHD inhibitors to stimulate angiogenesis in the periodontium.

Material and methods

Cell culture

GF and PDLF were prepared from extracted third molars after informed consent was obtained (Ethics Committee of the Medical University Vienna, #631/2007). The donors were selected on the basis of an absence of previous history of periodontal inflammation. Soft tissue particles of the gingiva from the tooth neck and of the periodontal ligament from the tooth root were scraped off the extracted teeth and explant cultures were performed separately. GF and PDLF were cultured in a humidified atmosphere at 37°C in α-minimal essential medium (αMEM; Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (PAA Laboratories, Linz, Austria), 100 U/mL penicillin G, and 100 μg/mL streptomycin (Invitrogen Corporation). In total, six strains of GF and seven strains of PDLF were established. For the experiments, fibroblasts (from randomly selected donors) that had not undergone more than 10 passages were used. For all experiments, cells were plated at a density of 50,000 cells/cm^2.

Viability, proliferation, and protein synthesis assays

Cells were incubated for 24 h with DMOG, DFO, L-MIM and $CoCl_2$ at a concentration range of 0.01–1 mM. To measure viability, cells were incubated with 1 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St Louis, MO, USA) for 2 h at 37°C. The MTT solution was removed and formazan crystals were solubilized with dimethyl sulfoxide. The absorbance (*A*) was measured at 550 nm with a photometer. For proliferation and protein synthesis, GF and PDLF were pulse-labelled with [^{3}H]thymidine and [^{3}H]leucine (both 0.5 μCi/well; Amersham Pharmacia Biotech, Bucks., UK), respectively, for the last 6 h of exposure to the PHD inhibitors. The plates were then subjected to liquid scintillation counting (Packard, Meriden, CT, USA). Data were normalized to unstimulated controls. We based the concentration of PHD inhibitors for further experiments on the results obtained from the viability, proliferation and protein synthesis tests.

Western blot

For HIF-1α and p27, periodontal fibroblasts in six-well plates were stimulated for 24 h with 0.1 mM DMOG, 0.3 mM DFO, 1.0 mM L-MIM and 0.1 mM $CoCl_2$. Cells were lysed in sodium dodecyl sulfate (SDS) buffer [62.5 mM Tris–HCl (pH 6.8 at 25°C), 2% (w/v) SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% (w/v) Bromophenol blue] containing protease inhibitors. Cell extracts were separated by SDS–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Amersham). Membranes were blocked in Tris-buffered saline containing 0.1% Tween-20 and 5% (w/v) nonfat dry milk. Anti-HIF-1α antibody (H-206; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p27 antibody (C-19; Santa Cruz Biotechnology) and

anti-actin antibody (#C-11; Santa Cruz Biotechnology) were diluted 1:1000 in Tris-buffered saline containing 0.1% Tween-20 and 5% bovine serum albumin and incubated overnight at 4°C. The primary antibody was detected with the appropriate secondary antibody (Dako, Glostrup, Denmark), using the enhanced chemiluminescence (ECL) method (GE Healthcare, Iittle Chalfont, UK). The ECL method was performed following the protocol of the manufacturer. In brief, the membranes were incubated with the ECL solution (GE Healthcare). The horseradish peroxidase bound to the secondary antibody catalyzes the oxidation of luminol. This reaction leads to the emission of light, which was then visualized using a chemiluminescence film (GE Healthcare).

For p38 and JNK, periodontal fibroblasts in six-well plates were stimulated with the PHD inhibitors for 24 h with and without the addition of 10 ng/mL of IL-1 for the last 20 min. Target proteins were detected by anti-JNK antibody (C-17; Cell Signaling Technology, Beverly, MA, USA), anti-phospho-JNK antibody (clone no. 9251; Cell Signalling) or anti-phospho-p38 antibody (clone no. 9211; Cell Signalling) and anti-actin antibody (C-11; Santa Cruz Biotechnology).

One-step quantitative RT-PCR analysis

Cellular RNA from two donors of GF and from two donors of PDLF was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and

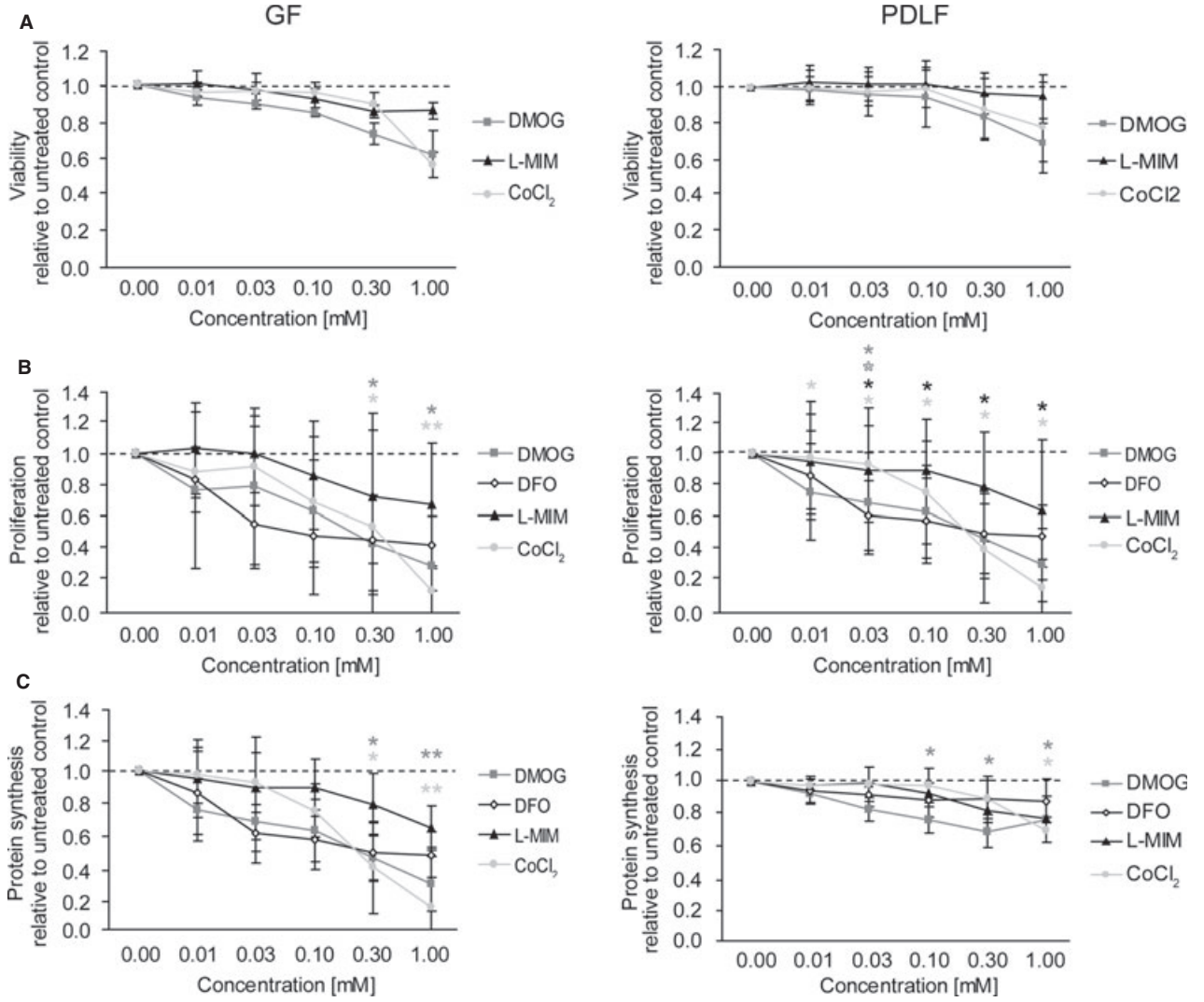

Fig. 1. Prolyl hydroxylase (PHD) inhibitors reduce viability, proliferation and protein synthesis in periodontal fibroblasts. To examine a possible cytotoxic effect of the PHD inhibitors dimethyloxallyl glycine (DMOG), desferrioxamine (DFO), L-mimosine (L-MIM) and $CoCl_2$, gingival fibroblasts (GF) and periodontal ligament fibroblasts (PDLF) were incubated with or without PHD inhibitors at 0.01–1 mM. (A) Cell viability was measured by detection of formation of formazan crystals. (B, C) Proliferation and protein synthesis were measured based on the incorporation of [^{3}H]thymidine and [^{3}H]leucine, respectively. Data points represent the mean ± standard deviation relative to the untreated control. Two independent experiments were performed with cells from three donors. The dashed line represents the levels of the untreated control. The color of the symbols represents the PHD inhibitor. *$p < 0.05$, **$p < 0.01$.

treated with DNAse I following the manufacturer's instructions (Invitrogen Corporation). RT and PCR were performed in a one-step methodology (SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR Kit; Invitrogen Corporation), according to the instructions of the manufacturer, on a 7000 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The following primers were used (20,21): (5′–3′) VEGF forward, CT ACCTCCACCATGCCAAGTG and VEGF reverse, TGATTCTGCCCTCC TCCTTCT; beta-actin forward, GC ATCCCCCAAAGTTCACAA and beta-actin reverse, AGGACTGGGC CATTCTCCTT. Amplification was performed as follows: one cycle of 50°C for 3 min; one cycle of 95°C for 5 min; 40 cycles of 95°C for 15 s, and 60°C for 30 s; and a final cycle of 40°C for 1 min. The $\Delta\Delta C_t$ method was used to calculate the relative levels of transcripts.

Immunoassays

Periodontal fibroblasts in 48-well plates were stimulated for 24 h with 0.1 mM DMOG, 0.3 mM DFO, 1.0 mM L-MIM and 0.1 mM $CoCl_2$, in the presence and absence of 10 ng/mL of IL-1. To analyze the release of VEGF and IL-6 into the cell-culture medium, ELISA kits were used according to the description of the manufacturer (Peprotech, Hamburg, Germany). Experiments on VEGF were also performed in the presence of SP600125 (a JNK inhibitor; Calbiochem, San Diego, CA, USA) and SB203580 (a p38 inhibitor; Sigma), both at 10 μM.

Immunohistochemistry

Samples were fixed in neutral-buffered formalin solution, and immunohistochemical procedures were performed as previously described (22). Briefly, cells were incubated with primary antibodies against Ki67 (MIB-I; 1:100 dilution; Dako) or HIF-1α (H-206; 1:250 dilution; Santa Cruz Biotechnology) at room temperature for 1 h, washed with phosphate-buffered saline and then detected with the appropriate biotinylated secondary antibody. The antibody was visualized using the ABC reagent and 3,3′-diaminobenzidine (DAB) solution (Vector Laboratories, Burlingame, CA, USA).

Statistical analysis

Data were compared using Kruskal–Wallis and post-hoc Mann–Whitney *U*-tests. Significance was assigned at the $p < 0.05$ level.

Results

PHD inhibitors reduce viability, proliferation, and protein synthesis

We first incubated periodontal fibroblasts with PHD inhibitors and assessed viability, proliferation, and protein synthesis. Overall, PHD inhibitors weakly reduced viability, but substantially reduced proliferation and protein synthesis (Fig. 1A–C). The MTT toxicity assay could not be reliably used for DFO because of an increase in the absorbance with high concentrations of DFO, even in the absence of cells (data not shown). However, DMOG, DFO, L-MIM and $CoCl_2$ reduced proliferation and protein synthesis. L-MIM showed the weakest effects on both proliferation and protein synthesis (Fig. 1B,C). Overall, GF and PDLF behaved similarly. The reduction of proliferation was paralleled by a reduction in Ki67 expression, as shown by immunohistochemistry (Fig. 2A). Furthermore, we found increased levels of the cell cycle protein p27 (Fig. 2B). These results suggest that when used at a

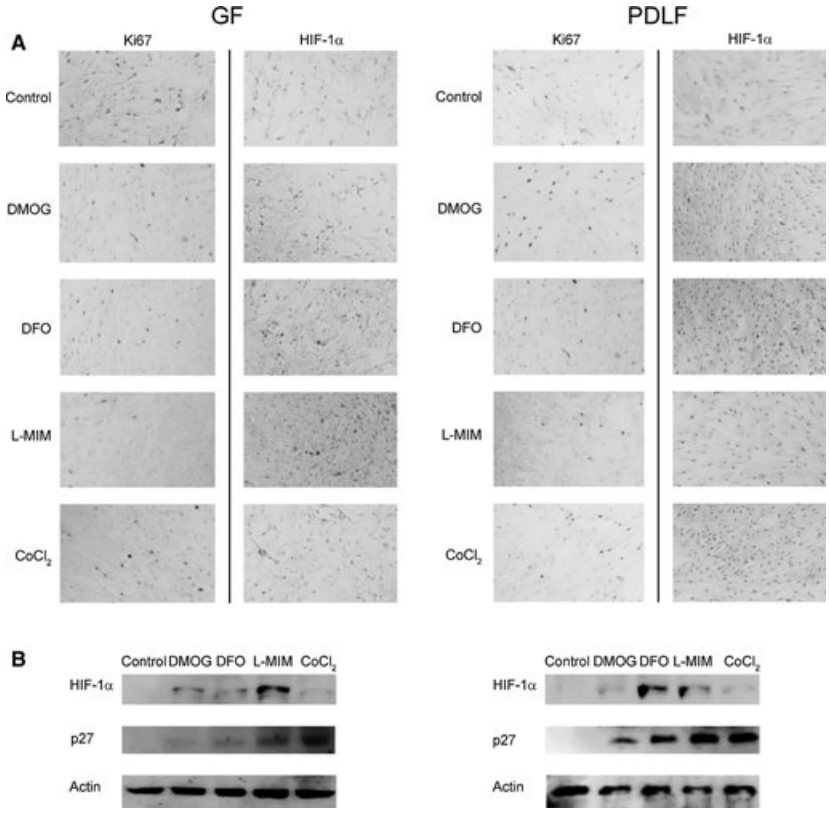

Fig. 2. Prolyl hydroxylase (PHD) inhibitors reduce the levels of Ki67 but increase the levels of hypoxia-inducible factor 1α (HIF-1α) and p27. To test whether PHD inhibitors can reduce the level of Ki67 and increase the levels of HIF-1α and p27 in periodontal fibroblasts, gingival fibroblasts (GF) and periodontal ligament fibroblasts (PDLF) were exposed to 0.1 mM dimethyloxallyl glycine (DMOG), 0.3 mM desferrioxamine (DFO), 1.0 mM L-mimosine (L-MIM) and 0.1 mM $CoCl_2$. (A) Immunohistochemical analyses of GF and PDLF to evaluate Ki67 and HIF-1α expression. (B) Western blotting of cell lysates to evaluate HIF-1α and p27 concentrations. As a control, western blots for actin were performed.

nontoxic concentration, DMOG, DFO, L-MIM and $CoCl_2$ can reduce proliferation and arrest cells in G0 (23). In order to avoid toxic effects on the fibroblasts we selected the concentration of the PHD inhibitors according to the results of the viability, proliferation and protein synthesis tests. For further experiments we used DMOG at 0.1 mM, DFO at 0.3 mM, L-MIM at 1 mM and $CoCl_2$ at 0.1 mM.

PHD inhibitors increase the production of VEGF by periodontal fibroblasts

Next we assessed the ability of PHD inhibitors to stimulate the production of VEGF. Evaluation of the intracellular levels of HIF-1α showed that PHD inhibitors increase the levels of the transcription factor, as shown by immunohistochemistry and western blotting (Fig. 2B). This increase of HIF-1α was paralleled by the expected increase in the production of VEGF (Fig. 3A). DMOG, DFO and L-MIM, but not $CoCl_2$, elevated VEGF levels in GF and PDLF also at the mRNA level (Table 1). Together, these results show that PHD inhibitors increase the production of VEGF by GF and PDLF. Overall, GF and PDLF behaved similarly.

We then evaluated whether PHD inhibitors also increase the production of VEGF in the presence of IL-1. This was indeed the case, as DMOG increased the production of VEGF compared to fibroblasts treated with IL-1 alone (Fig. 3B). In addition, the increase in VEGF production was observed in the presence of JNK and p38 signaling inhibitors (Table 2). Thus, PHD inhibitors act independently from IL-1 and the activation of JNK in GF and PDLF.

PHD inhibitors do not provoke or reduce an inflammatory response

We next determined if PHD inhibitors can affect the expression of IL-6, a typical inflammatory cytokine (Figs 4 and 5). The PHD inhibitors did not reduce the IL-1-induced expression of IL-6 and also did not modulate the phosphorylation of JNK and p38 under these conditions (Fig. 4A,B). We then measured the basal production of IL-6 in response to PHD inhibitors (Fig. 5A). The IL-6 levels were not significantly increased in GF and PDLF. Interestingly, cells from individual donors showed a strong response to PHD inhibitors (Fig. S1). Moreover, none of the PHD inhibitors stimulated the phosphorylation of JNK and p38 to the same extent as did IL-1 (Fig. 5B). Overall, similar results were obtained for fibroblasts from the gingiva and from the periodontal ligament. These results showed that PHD inhibitors have neither a significant proinflammatory nor anti-inflammatory effect in this *in vitro* model.

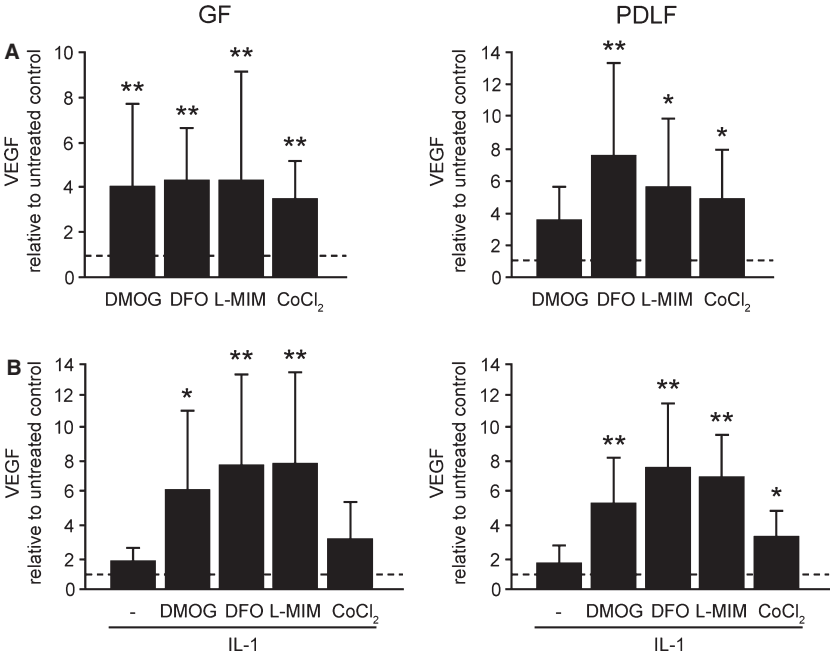

Fig. 3. Prolyl hydroxylase (PHD) inhibitors stimulate the release of vascular endothelial growth factor (VEGF) from periodontal fibroblasts. To examine whether the PHD inhibitors stimulate the production of VEGF, gingival fibroblasts (GF) and periodontal ligament fibroblasts (PDLF) were incubated with 0.1 mM dimethyloxallyl glycine (DMOG), 0.3 mM desferrioxamine (DFO), 1.0 mM L-mimosine (L-MIM) and 0.1 mM $CoCl_2$, either alone (A) or in the presence of interleukin-1 (IL-1) (B). Then, the concentration of VEGF was measured in the cell culture supernatants. Bars represent the mean + standard deviation relative to the untreated control. Untreated cells (dashed line) and cells treated with IL-1 (-) served as controls. Three independent experiments were performed with cells from three donors to determine the basal VEGF production. Statistical difference was calculated compared with the untreated control. For the VEGF production in the presence of IL-1, two independent experiments were performed with cells from three donors. Statistical difference was calculated compared with the IL-1-treated cells. $*p < 0.05$, $**p < 0.01$.

Table 1. VEGF expression in response to PHD inhibitors

PHD inhibitors	GF	PDLF
DMOG	3.03 ± 0.59	2.59 ± 0.31
DFO	7.90 ± 0.19	3.96 ± 1.14
L-MIM	7.91 ± 0.53	7.83 ± 2.61
$CoCl_2$	0.64 ± 0.69	0.71 ± 1.14

To examine whether the prolyl hydroxylase (PHD) inhibitors dimethyloxallyl glycine (DMOG), desferrioxamine (DFO), L-mimosine (L-MIM) and $CoCl_2$ stimulate the expression of vascular endothelial growth factor (VEGF), gingival fibroblasts (GF) and periodontal ligament fibroblasts (PDLF) were incubated for 24 h with 0.1 mM DMOG, 0.3 mM DFO, 1.0 mM L-MIM and 0.1 mM $CoCl_2$. RNA was isolated and the relative expression of *VEGF* was measured by quantitative PCR. Data are given as mean ± standard deviation relative to the untreated control. The experiment was performed with GF from two donors and with PDLF from two donors.

Table 2. Pharmacological inhibition of JNK and p38 signaling and VEGF production in response to PHD inhibitors

	GF			PDLF		
Inhibitors	W/O	SP600125	SB203580	W/O	SP600125	SB203580
DMOG	1.93 ± 0.37	1.48 ± 0.28	1.71 ± 0.42	2.27 ± 2.08	2.15 ± 2.94	3.11 ± 3.91
DFO	3.16 ± 0.57	2.77 ± 0.49	2.46 ± 0.23	10.24 ± 6.77	9.62 ± 6.84	6.80 ± 4.58
L-MIM	2.65 ± 0.43	2.11 ± 0.38*	1.95 ± 0.21*	7.13 ± 4.71	7.99 ± 6.47	5.53 ± 3.78
$CoCl_2$	2.57 ± 0.40	2.05 ± 0.19	2.16 ± 0.38	6.25 ± 3.58	6.93 ± 5.66	4.52 ± 3.07

To examine whether the prolyl hydroxylase (PHD) inhibitors dimethyloxallyl glycine (DMOG), desferrioxamine (DFO), L-mimosine (L-MIM) and $CoCl_2$ stimulate the production of vascular endothelial growth factor (VEGF) involving the mitogen-activated kinases c-Jun N-terminal kinase (JNK) and p38, gingival fibroblasts (GF) and periodontal ligament fibroblasts (PDLF) were incubated with 0.1 mM DMOG, 0.3 mM DFO, 1.0 mM L-MIM and 0.1 mM $CoCl_2$ for 24 h, with and without (W/O) the addition of inhibitors of JNK (SP600125) and p38 (SB203580). Data are given as mean ± standard deviation relative to the untreated control. Two independent experiments were performed with GF from three donors and with PDLF from two donors. *$p < 0.05$.

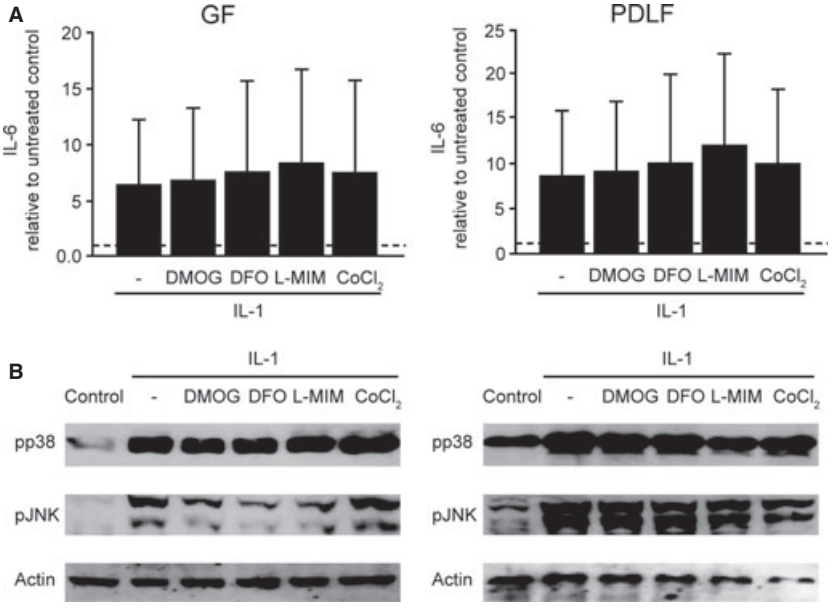

Fig. 4. Prolyl hydroxylase (PHD) inhibitors do not induce anti-inflammatory responses. (A) To test whether PHD inhibitors can decrease the inflammatory response to interleukin (IL)-1, gingival fibroblasts (GF) and periodontal ligament fibroblasts (PDLF) were exposed to 0.1 mM dimethyloxallyl glycine (DMOG), 0.3 mM desferrioxamine (DFO), 1.0 mM L-mimosine (L-MIM) and 0.1 mM $CoCl_2$, with and without 10 ng/mL of IL-1. Untreated cells (dashed line) and cells treated with IL-1 alone (-) served as controls. Cell supernatants were then subjected to immunoassays for IL-6. Bars represent the mean + standard deviation relative to the untreated control. The dashed line represents the levels of the untreated control. Three independent experiments were performed with cells from three donors. The results did not reach statistical significance ($p > 0.05$). (B) To test whether PHD inhibitors can modulate the phosphorylation of c-Jun N-terminal kinase (JNK) and p38, the mitogen-activated kinases which are involved in the inflammatory response in GF and PDLF were exposed to 0.1 mM DMOG, 0.3 mM DFO, 1.0 mM L-MIM and 0.1 mM $CoCl_2$ for 24 h with the addition of 10 ng/mL of IL-1 for the last 20 min. Untreated cells served as controls (Control). Then, the cells were lysed with sodium dodecyl sulfate (SDS) buffer and the phosphorylation of JNK and p38 (pJNK and pp38, respectively) was evaluated by western blotting. As a control, western blots for actin were performed.

Discussion

Preclinical studies revealed that inhibition of oxygen sensors by PHD inhibitors supports bone regeneration and wound healing (11,12). In the present study we investigated the impact of PHD inhibitors on cells of the periodontium. We found that PHD inhibitors reduced cell proliferation at concentrations where cell viability was virtually unaffected. At these concentrations, PHD inhibitors increase the production of VEGF in periodontal fibroblasts. This was also observed under inflammatory conditions. While PHD inhibitors stimulate VEGF production, our results suggest that PHD inhibitors did not substantially modulate IL-1-induced and basal IL-6 production, and p38/JNK signaling.

In agreement with the results for other cell types, including smooth muscle cells and hepatocytes, we found that PHD inhibitors reduced proliferation in periodontal cells (24–26). A reduction in proliferation does not necessarily mean that cell viability is impaired. PHD inhibitors can induce growth arrest by pausing the cell cycle (26,27). Our finding that PHD inhibitors decrease intracellular levels of Ki67, which is observed in G0 arrest, supports the assumption that PHD inhibitors can pause the cell cycle in periodontal cells (23). In line with this observation we found increased levels of p27, which is a negative regulator of the cell cycle (26,27). Thus, PHD inhibitors presumably arrest the cell cycle, while periodontal fibroblasts remain viable and produce VEGF. This suggestion is supported by a previous study where growth arrest involves p27 but not HIF-1α (26,27). Also, our findings that PHD inhibitors increase the expression of VEGF are in line with studies on osteoblasts (12), osteoclasts (28), endothelial cells (15) and epithelial cells (15). Interestingly,

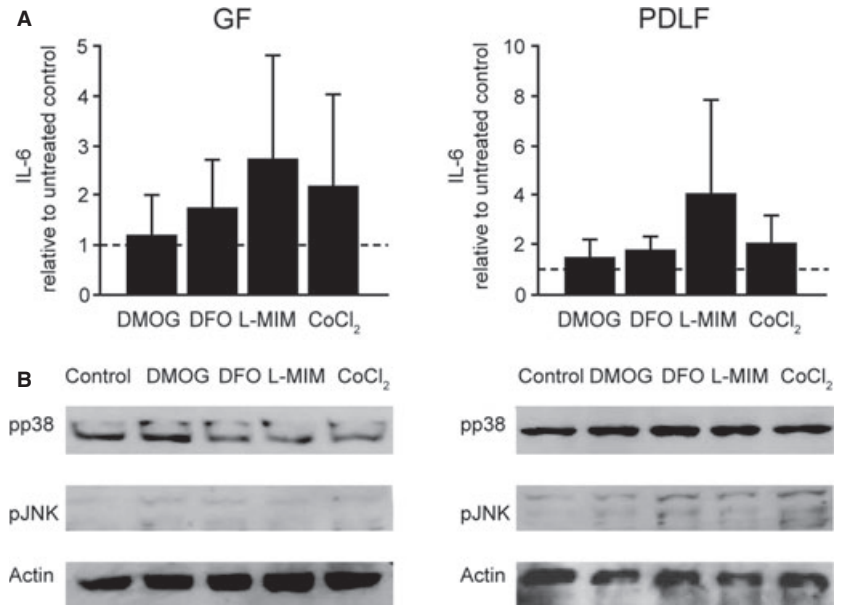

Fig. 5. Prolyl hydroxylase (PHD) inhibitors do not induce significant proinflammatory responses. (A) To test whether PHD inhibitors can increase the basal production of inflammatory cytokines of periodontal fibroblasts, gingival fibroblasts (GF) and periodontal ligament fibroblasts (PDLF) were exposed to 0.1 mM dimethyloxallyl glycine (DMOG), 0.3 mM desferrioxamine (DFO), 1.0 mM L-mimosine (L-MIM) and 0.1 mM $CoCl_2$. Untreated cells (dashed line) served as controls. Cell supernatants were then subjected to immunoassays for IL-6 (A). Bars represent the mean + standard deviation relative to the untreated control. The dashed line represents the levels of the untreated control. Three independent experiments were performed with cells from three donors. The results did not reach statistical significance ($p > 0.05$). (B) To test whether PHD inhibitors can modulate the phosphorylation of c-Jun N-terminal kinase (JNK) and p38, the mitogen-activated kinases that are involved in the inflammatory response GF and PDLF were exposed to 0.1 mM DMOG, 0.3 mM DFO, 1.0 mM L-MIM, and 0.1 mM $CoCl_2$. Untreated cells served as controls (Control). Then the cells were lysed with sodium dodecyl sulfate (SDS) buffer and the phosphorylation of JNK and p38 (pJNK and pp38, respectively) was evaluated by western blotting. As a control, western blots for actin were performed.

treatment with $CoCl_2$ slightly elevated the levels of VEGF protein but not the levels of *VEGF* mRNA in our setting. The mechanism of this response is unknown. Overall, no substantial differences were observed between GF and PDLF. Together these data suggest that PHD inhibitors target a highly conserved signaling pathway that increases the production of VEGF (12,14,15,29–31). However, for the application of PHD inhibitors in periodontology it is important to understand the response of cells in the presence of inflammatory factors such as IL-1. It is known that IL-1 can increase HIF-1α levels in gingival fibroblasts (32). However, our finding that VEGF production in response to PHD inhibitors occurs in the presence of IL-1 is novel. Thus, cells of the periodontium are a promising target for a new therapeutic approach that utilizes PHD inhibitors to stimulate periodontal regeneration.

Questions arise regarding whether PHD inhibitors can modulate inflammation. Here we show that PHD inhibitors failed to modulate IL-1-induced IL-6 production and p38/JNK signaling, suggesting that PHD inhibitors do not have a relevant anti-inflammatory function. No similar studies on anti-inflammatory functions have yet been reported. However, numerous studies on a potential pro-inflammatory function of PHD inhibitors are available. For example, in endothelial cells, $CoCl_2$ induced IL-8 expression via the activation of p38 (16). In epithelial cells, DFO activates p38, JNK and IL-6 production (19) and $CoCl_2$ increased IL-6 in neuronal cells (17). In contrast, DMOG decreased IL-8 expression in endothelial cells, suggesting that PHD inhibitors might also exert anti-inflammatory functions (18). The present study could not support either of these contrasting findings. However, PHD inhibitors substantially increased IL-6 production in cells from individual donors, but the reasons for this remain unknown. Therefore, more research on the pro-inflammatory and anti-inflammatory effects of PHD inhibitors is necessary.

Many other questions remain unanswered. Angiogenesis is a complex process that does not exclusively depend on the expression of VEGF. Thus, our *in vitro* findings do not necessarily mean that VEGF in the cell culture supernatant can also stimulate the formation of tubular structures of endothelial cells or enhance angiogenesis in the chorioallantoic membrane assay. Another limitation is the complex cellular composition of the periodontium, which cannot be recapitulated under *in vitro* conditions. We do not know whether the PHD inhibitors stimulate angiogenesis in the periodontium and if that leads to successful regeneration. More VEGF may not be better. While VEGF and angiogenesis play an essential role in the repair and regeneration of the damaged tissue (1,2), both also have a pathological role in the onset and the progression of tissue destruction (1,4,33). It is thus clear that angiogenesis can have positive and negative effects and it is reasonable that this is also true for PHD inhibitors. Further studies are needed to answer this question.

Clinically, periodontitis is associated with increased VEGF and HIF-1α levels, which raises the question of whether stabilization of HIF-1α by PHD inhibitors is a feasible approach to stimulate periodontal regeneration (4,34,35). In addition, HIF-1α has been shown to induce the expression of RANKL, which is an essential factor in osteoclastogenesis (36). As both angiogenesis and increased osteoclastogenesis are involved in the progression of periodontitis, it is important to answer the question of how PHD inhibitors modulate the progression of periodontitis. We also

cannot rule out the potential side effects of PHD inhibitors as they reduce proliferation and may also have other activities (10). Persistent stimulation of angiogenesis may exacerbate the formation of granulation tissue and delay the late stages of wound healing (37,38). However, studies assessing the effect of local application of PHD inhibitors on bone and soft tissue regeneration show promising results (11,12,31). To circumvent any possible indirect effects on the inflammatory response, PHD inhibitors might be applied when inflammation has ceased. It is important that further studies address the effect on the kinetics of periondontal tissue regeneration.

What we have contributed is to show that PHD inhibitors can effectively increase the production of VEGF by fibroblasts from the gingiva and the periodontal ligament while not interfering with the inflammatory response *in vitro*. The novel findings of this proof-of-principle study should stimulate further research on the potential clinical application of PHD inhibitors in periodontology.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Donor variations in the production of IL-6 under the presence of PHD inhibitors.

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