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Article

Palmitate-triggered COX2/PGE2-mediated hyperinflammation in dual-stressed PdL fibroblasts is mediated by repressive H3K27 trimethylation.

Lisa Schuldt [1], Michael Reimann [2], Katrin von Brandenstein [1], Julia Steinmetz [1], Annika Döding [2], Ulrike Schulze-Späte [2], Collin Jacobs [3] and Judit Symmank [1,*]

[1] Department of Orthodontics, University Hospital Jena, Leutragraben 3, 07743 Jena, Germany
[2] Section of Geriodontics, Department of Conservative Dentistry and Periodontics, University Hospital Jena, Leutragraben 3, 07743 Jena,
[3] Department of Orthodontics, Center for Dental, Oral and Maxillofacial Medicine, University Hospital Jena, An der Alten Post 4, 07743 Jena, Germany
* Correspondence: judit.symmank@med.uni-jena.de

Abstract: The interrelationship between periodontal disease, obesity-related hyperlipidemia and mechanical forces as well as their modulating effects on the epigenetic profile of periodontal ligament (PdL) cells are assumed to be remarkably complex. The PdL serves as connective tissue between teeth and alveolar bone for pathogen defense and inflammatory response to mechanical stimuli occurring during tooth movement. Altered inflammatory signaling could promote root resorption and tooth loss. Hyperinflammatory COX2/PGE2 signaling was reported for human PdL fibroblasts (HPdLF) concomitantly stressed with *P. gingivalis* lipopolysaccharides and compressive force after exposure to palmitic acid (PA). The aim of this study was to investigate to what extent this is modulated by global and gene-specific changes in histone modifications. Quantitative expression of epigenetic key players and global H3Kac as well as H3K27me3 levels were evaluated in dual stressed HPdLF exposed to PA revealing a decreased force-related reduction in repressive H3K27me3. UNC1999-induced H3K27me3 inhibition reversed the hyperinflammatory response of dual-stressed PA-cultures characterized by *COX2* expression, PGE2 secretion and THP1 adhesion. Reduced expression of the anti-inflammatory cytokine IL10 and increased association of H3K27me3 at its promoter-associated sites were reversed by inhibitor treatment. Thus, the data highlight an important epigenetic interplay between different stimuli to which the PdL is exposed.

Keywords: Periodontitis, Tooth movement, Obesity, Palmitic Acid, Histone Modification, Inflammation, COX2/PGE2, IL10

1. Introduction

Genetic predisposition, unfavorable environmental conditions and an unhealthy lifestyle are important risk factors for the onset and progression of periodontal disease, but cannot fully account for individual susceptibility [1]. In view of the high prevalence of this chronic, non-communicable disease and subsequent economic health care system implications [2], the investigation of additional key regulatory mechanisms has been in focus of recent periodontitis research. This has drawn attention to epigenetic regulatory mechanisms as important factors in the pathogenesis of the disease [3]. Epigenetic modifications include in part heritable changes on DNA and histones that restrict the accessibility of DNA to the transcription machinery without altering the genome [4]. With regard to the inflammatory aspects of periodontal disease, of these alterations, DNA methylation and post-translational modifications (PTM) to histone tail amino acids have been thoroughly investigated [5]. PTMs at histones include acetylation and methylation, which modulate transcriptional activity in a context-dependent manner [6]. While the attachment of acetyl groups by histone acetyltransferases (HAT) is generally associated with an

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opening of chromatin structure and a beneficial environment for gene expression, the effects of histone methylation depend on the appropriately modified amino acid and the abundance of methyl groups [6,7]. For example, trimethylation of lysines at position 27 (K27) on histone 3 (H3) has been linked to transcriptional repression, while H3K4 trimethylation is associated with gene activation [7]. Regarding the expression of key factors regulating PTMs, a number of comparative studies showed differences between periodontal patients and healthy subjects [5], presumably caused by the pathogenic infection and possibly linked to an excessive presence of bacterial metabolites such as short-chain fatty acids [8].

Elevated serum levels of long-chain fatty acids such as saturated palmitic acid (PA) are typically associated with obesity and have been investigated for its role in disease-related low-grade systemic inflammation [9-11]. Further, a pro-inflammatory characteristic of PA has been shown under hyperlipidemic conditions [12-20], although in general fatty acids are relevant for normal cell functions [21]. Several studies reported an interrelationship between both diseases. For example, a delayed response to the infection with gram-negative oral anaerobe *Porphyromonas gingivalis* (*P. gingivalis*) was reported for obese mice [22]. *P. gingivalis* has been described as a keystone pathogen affecting oral health and disease, possibly due to its unique ability to evade the host immune response [23]. It has been demonstrated that a variety of virulence factors such as lipopolysaccharides (LPS), gingipains and fimbriae are responsible for its pathogenity [24]. In animal studies, enhanced swallowing of *P. gingivalis* was reported to induce changes in gut microbiome, increase systemic inflammation and induce metabolic alterations [25,26]. In addition, adipocytes stimulated with *P. gingivalis*-LPS exhibited a more pronounced pro-inflammatory profile, indicating a potential contributing factor to the pro-inflammatory state associated with obesity [27,28]. Although various changes in the host oral environment and bacterial biofilm composition are required for the onset and progression of periodontitis, the pro-inflammatory impact of *P. gingivalis*-LPS is commonly used to mimic periodontitis-causing conditions *in vitro*.

Even though inflammatory processes are increased in both diseases, their concomitant impact on the inflammatory response of the periodontal ligament (PdL) to mechanical forces such as during trauma, mastication or orthodontic tooth movement is still poorly studied. The PdL is the connective tissue between teeth and alveolar bone, and its most abundant cell type, PdL fibroblasts, modulate the transient, aseptic inflammatory response to compressive and tensile forces in a temporal and spatial manner [29-31]. Alterations in their inflammatory response may increase the risk of tooth root resorption and tooth loss, primarily by affecting tissue and bone remodeling cells [32,33]. In rats suffering from periodontitis, up-regulated expression of several cytokines in the PdL and enhanced presence of activated osteoclasts were observed after tooth movement in addition to an increased extent of dorsal root resorption [34]. However, an additional impact of obesity-related changes on tooth movement has not yet been investigated in either animal or human studies. In view of the tremendous heterogeneity of obesity-associated cellular adaptions, *in vivo* studies can be quite challenging to conduct. Thus, even the results of prospective controlled clinical trials examining only the effects of increased BMI on orthodontic tooth movement (OTM) are conflicting, reporting both increased and decreased rates of OTM [35-38].

However, influences on epigenetic regulatory mechanisms are evident with regards to high-fat diets and obesity-associated alterations [39]. In this line, increased histone acetylation was reported in several cell types under hyperlipidemic conditions, potentially due to the role of fatty acids as lipid-based alternative donors for acetyl-CoA that is required for this epigenetic mark [21,40]. In addition, a potential influence of PA on repressive methylated histone marks has recently been reported [41,42]. This could provide a framework for the interplay with periodontitis-induced changes and a target for potential therapeutic interventions, as epigenetic inhibitors are already widely used in treatment of a variety of diseases [43].

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We recently reported that with PA incubated human periodontal ligament fibroblasts (HPdLF) showed excessive inflammatory response to simultaneous stimulation with *P. gingivalis*-LPS and compressive force mainly through enhanced secretion of prostaglandin E2 (PGE2) [44], which is regulated by cyclooxygenase 2 (COX2) [45]. The aim of this study was to investigate whether this excessive inflammation is based on epigenetic alterations induced by palmitic acid.

2. Materials and Methods

2.1 Cell culture

Culture medium consisting of Dulbecco's modified Eagle medium (DMEM; Thermo Fisher Scientific, Carlsbad, CA, USA) containing 4.5 g/L glucose, 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Carlsbad, CA, USA),), 100 U/mL penicillin, 100 µg/mL streptomycin, and 50 mg/L L-ascorbic acid were used for growing commercially acquired human periodontal ligament fibroblast (HPdLF, Lonza, Basel, Switzerland) at 37 ◦C, 5% CO2 and 95% humidity. HPdLF were passaged at a confluency of 75% with 0.05% Trypsin/EDTA (Thermo Fisher Scientific, Carlsbad, CA, USA) and used for experiments at passage four to eight.

RPMI 1640 medium (Thermo Fisher Scientific, Carlsbad, CA, USA) containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin was used for culturing THP1 cells (DMSZ, Braunschweig, Germany) at 37 °C, 5% CO2 and 95% humidity. Weekly, passages were performed and 1 x 10^6 cells were seeded into new T175 culture flask for further culturing (Thermo Fisher Scientific, Carlsbad, CA, USA).

2.2 Stimulation with Palmitic Acid

For the analysis of RNA expression, 2.5 × 10^4 HPdLF were seeded into each well of a 6-well plate. For immunofluorescence staining, 5 × 10^3 cells were plated onto coverslips into each well of a 48-well-plate. Cells were cultured for 24 h in DMEM culture medium prior six-day stimulation with 200 µM palmitic acid (PA). PA containing media was produced as described previously [44]. Briefly, dried PA was dissolved at 70 ◦C in sterile water containing 50 mM NaOH, complexed with 37 ◦C preheated bovine serum albumin (BSA, Seqens IVD, Limoges, France) and added to DMEM culture medium. BSA-containing medium was used for control stimulation.

2.3 Stimulation with P. gingivalis Lipopolysaccharides

To simulate pathogenic stimulation, 10 µg/mL lipopolysaccharids (LPS) of *Porphyromonas gingivalis* (*P. gingivalis*; InvivoGen, San Diego, CA, USA) were applied to the culture medium for six hours simultaneous to compressive force application.

2.3 UNC1999 application

For inhibition of H3K27 trimethylation, different concentrations of the EZH1/EZH2 inhibitor UNC1999 (0,5 µM, 1.0 µM and 1.5 µM) were applied for 24 hours on 75% confluent HPdLF. For further treatment 1.5 µM UNC1999 was applied for six hours simultaneous to *P. ginigivalis* LPS and compressive force application.

2.4 Application of compressive force

A compressive force of 2 g/cm² was applied according to the protocol of Kirschneck *et al.* [46] and as previously reported [47]. Briefly, simulataneous to LPS application, glass plates were placed on fatty acid cultured HPdLF for six hours at 37 °C, 5% CO_2 and 95 % humidity. Afterwards cells were either directly isolated with TRIzol Reagent (Thermo Fisher Scientific, Carlsbad, CA, USA) for expression analysis or with Dulbecco's Phosphate-Buffered Solution (DPBS, Gibco) for histone extraction and subsequently protein analysis.

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In 48-well plates the compressive force was applied via six-hour centrifugation at 30 °C with a force of 7.13 g/cm². Control cells were cultured at 30 °C for the duration of force application.

2.5 Expression analysis with quantitative PCR

RNA isolation, cDNA synthesis and quantitative PCR were performed as previously described [48]. Primer sequences used for target amplification are shown in **Table 1**.

Table 1: qPCR primer sequences of human genes indicated in 5`-3` direction. bp, base pairs; fw, forward; Length, amplicon length; PRC, polycomp repressive complex; rev, reverse.

Gene	Gene Symbol	NCBI Gene ID	Primer Sequence	Length
Prostaglandin-endoperoxide synthase 2	*PTGS2 (COX2)*	5743	fw: GATGATTGCCCGACTCCCTT rev: GGCCCTCGCTTATGATCTGT	185 bp
Embryonic ectoderm development	*EED*	8726	fw: TGCGATGGTTAGGCGATTTG rev: CCAAATGTCACACTGGCTGT	158 bp
Enhancer of zeste 1 PRC2 subunit	*EZH1*	2145	fw: CGAGAATGTGACCCTGACCT rev: TTATGAAGGTGCCCCATCCG	154 bp
Enhancer of zeste 2 PRC2 subunit	*EZH2*	2146	fw: ACAGTTCGTGCCCTTGTGTG rev: CACTCTCGGACAGCCAGGTA	148 bp
Histone deacetylase 2	*HDAC2*	3066	fw: ACTGATGCTTGGAGGAGGTG rev: CTGGAGTGTTCTGGTTTGTCA	185 bp
Histone deacetylase 3	*HDAC3*	8841	fw: GCTGGGTGGTGGTGGTTATA rev: TTCTGATTCTCGATGCGGGT	174 bp
Interleukin 10	*IL10*	3586	fw: AGCCATGAGTGAGTTTGACA rev: AGAGCCCCAGATCCGATTTT	141 bp
Lysine acetyltransferase 8	*KAT8*	84148	fw: GCAAGATCACTCGCAACCAA rev: AGTCTTCGGGGAATGGTGAG	195 bp
Nuclear receptor coactivator 3	*NCOA3*	8202	fw: GGCTCTATTCCCACATTGCC rev: CCCAGTTGGTTAGATGCTGC	158 bp
Ribosomal protein L22	*RPL22*	6146	fw: TGATTGCACCCACCCTGTAG rev: GGTTCCCAGCTTTTCCGTTC	98 bp
SIN3 transcription regulator family member A	*SIN3A*	25942	fw: GAGCAGCAGGAAAAGGAAGG rev TGTAGACGCTTGCTTACACG	200 bp
TATA-box binding protein	*TBP*	6908	fw: CGGCTGTTTAACTTCGCTTCC rev: TGGGTTATCTTCACACGCCAAG	86 bp

2.6 Immunofluorescent staining

To detect trimethylated H3K27 after specific treatments, coverslips with cultured HPdLF were fixed in 4% PFA for 10 minutes, washed with phosphate-buffered saline (PBS and incubated with the primary antibody for 1.5 hours and the secondary antibody for 45 minutes. DAPI (Thermo Fisher Scientific, Carlsbad, CA, USA; 1:10000 in PBS) was used for nuclei staining. Following antibodies were used: Mouse-anti-human H3K9/14/18/23/27 (Thermo Fisher Scientific, Carlsbad, CA, USA; 1:500), Rabbit-anti-human H3K27me3 (Thermo Fisher Scientific, Carlsbad, CA, USA; 1:250), Goat-anti-mouse-Cy5 and goat-anti-Rabbit-Cy5 (Jackson ImmunoResearch, Baltimore Pike, PA, USA; 1:1000).

2.7 THP1 adhesion assay

To detected pro-inflammatory cytokine secretion by stimulated HPdLF, THP1 adhesion assay was performed as previously described [48]. Adhesive THP1 cell numbers were determined and displayed in relation to the total number of HPdLF per each image.

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2.8 MTT assay

Cell vitality was analyzed at the plate reader Infinite ® M Nano (TECAN, Männedorf, Swiss) with the MTT colorimetric assay (Sigma Aldrich, St. Louis, Missouri, USA) according to manufacturer`s protocol.

2.9 Nuclear extraction andhistone methyltransferase activity assay

EpiQuik Nuclear Extraction Kit (EpiGentek, Farmingdale, New York, USA) was used for nuclear extraction according to manufacturer`s protocol. The activities of histone methyltransferases specifically methylating H3K27 were analyzed with EpiQuik Histone Methyltransferase Activity/Inhibition Assay Kit H3K27 (EpiGentek, Farmingdale, New York, USA) according to manufacturer`s guidelines at the plate reader Infinite ® M Nano (TECAN, Männedorf, Swiss).

2.10 ELISA

Medium supernatant of stimulated HPdLF was isolated 24 hours after force application to ensure proper protein secretion. To analyzed PGE2 secretion, prostaglandin E2 ELISA (PGE2; R&D Systems, Minneapolis, MN, USA) was used according to the manufacturer`s guidelines.

2.11 Chromatin-Immunoprecipitation

To analyze the association of acetylated H3K at *IL10* gene regions, chromatin-immunoprecipitation (ChIP) was performed. First, DNA and protein were cross-linked with 1% formaldehyde in PBS for 10 min and neutralized with 120 mM glycine. After cell harvesting in ice-cold PBS and centrifugation for 5 min at 1000 x g at 4°C, an approximate cell number of 1×10^6 cells per tube was stored at -80 °C. ChIP was performed with the Zymo-Spin ChIP Kit (Zymo Research, Freiburg, Germany) according to manufacturer`s protocol with the ChIP-valuated antibodies rabbit-anti-human H327me3 (Thermo Fisher Scientific, Carlsbad, CA, USA) and mouse-anti-IgG (Thermo Fisher Scientific, Carlsbad, CA, USA). Throughout the ChIP process, one percent input controls were stored for later normalization. The amount of specific DNA fragments bound to H3K27me3 was determined as previously described [49]. Briefly, prior quantitative analysis with the qTOWER3 (Analytik Jena, Jena, Germany) according to the manufacturer`s protocol using Luminaris Color HiGreen qPCR Master Mix (Thermo Fisher Scientific, Carlsbad, CA, USA), primer-specific pre-amplification was performed. Primer sequences used for analysis are displayed in **Table 2**. Per cent-input method was used to normalize DNA content [50]. IgG control was subtracted from H3K27me3-specific sample.

Table 2 qPCR primer pairs located in the human *IL10* in promoter regions (#1, #2, #3) and a non-promoter region (#4) indicated in 5`-3` direction. bp, base pairs; fw, forward; Length, amplicon length; rev, reverse.

Location	Label	Primer Sequence	Length
promoter region	*#1*	fw: TGAAGAAGTCCTGATGTCAC rev: TTACCTATCCCTACTTCCCC	187 bp
promoter region	*#2*	fw: AGCACTACCTGACTAGCATA rev: AGAGACTGGCTTCCTACAG	192 bp
promoter region	*#3*	fw: GGGGACCCAATTATTTCTCA rev: TGGGCTACCTCTCTTAGAAT	188 bp
non-promoter region	#4	fw: GCTTAGAGCGTTTCCAGACC rev: CTCCCCACTGTAGACATCCA	131 bp

2.12 Microscopy, Image Analysis, and Statistics

THP1 adhesion assay, H3Kac and H3K27me3 staining was imaged with the inverted confocal laser scanning microscope TCS SP5 (Leica, Wetzlar, Germany). Fiji software (https://imagej.net/Fiji, accessed on 01.04.2017) was used to analyze THP1 cell numbers and fluorescence intensity. Graph Pad Prism (https://www.graphpad.com, accessed on

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01.02.2021) was used for statistical analysis. Adobe Photoshop CS5 (https://adobe.com, accessed on 01.02.2013) was used for figure illustration. One-way ANOVA and post hoc test (Tukey) were used as statistical tests. All experiments were independently repeated at least three times and examined in technical duplicates. Diagrams show mean ± SE. Significance levels: p value < 0.05 */#/§; p value < 0.01 **/##/§§; p value < 0.001 ***/###/§§§.

3. Results

3.1. Palmitic acid exposure impacts force-induced reduction of H3K27me3 in LPS-stimulated human periodontal ligament fibroblasts

Free fatty acids can serve as an alternative source of acetyl CoA and thus affect H3 lysine acetylation of human periodontal ligament fibroblasts [51]. To elucidate the role of H3K acetylation in the regulation of the hyper-inflammatory response of PA-exposed HPdLFs simultaneously stimulated by mechanical and bacterial-induced stress, we first examined the expression of relevant regulators. Quantitative PCR, however, revealed neither changes in genes encoding major histone acetyltransferase (*NCOA3*, *KAT8*), nor histone deacetylases (*HDAC2*, *HDAC3*) in dual stimulated PA cultures as compared to BSA controls (**Fig. 1a**). Additional quantitative analysis of immunofluorescently stained H3K9/14/18/23/27 acetylation showed increased levels due to compressive forces, however, no differences due to PA exposure were detected as well (**Fig. 1b, c**).

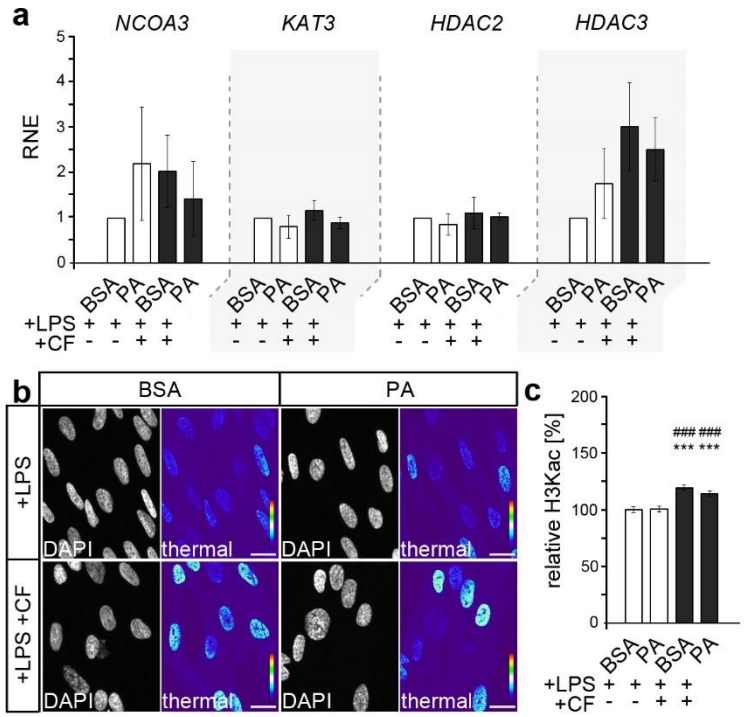

Figure 1: Palmitic acid did not impact H3Kac in compressed HPdLF stimulated with *P. gingivalis* LPS. (**a**) Quantitative expression analysis of genes encoding histone acetyl transferases *NCOA3* and *KAT3* and histone deacetylases *HDAC2* and *HDAC3* in human periodontal ligament fibroblasts (HPdLF) exposed to palmitic acid (PA) and simultaneous stimulated with compressive force (CF) and P. gingivalis-LPS as compared to BSA controls. (**b**, **c**) Representative microphotographs of global H3K9/14/18/23/27 acetylation (H3Kac) in HPdLFs treated with PA and co-stimulated with CF and LPS in comparison to BSA controls. Cell nuclei are labeled with DAPI and H3Kac staining intensity is shown in thermal LUT (thermal) analyzed in (c) in relation to BSA control. *** $p < 0.001$ in relation to BSA+LPS, ### $p < 0.001$ in relation to PA+LPS; One-Way ANOVA and post hoc test (Tukey). Scale bars: 10 μm in (b). RNE, relative normalized expression.

Considering that PA can also affect repressive histone methylation such as H3K27 trimethylation (H3K27me3) [41,42], we now investigated whether alterations in this modification could contribute to triggering the excessive inflammatory stress response. We

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also performed quantitative PCR on genes encoding components of the polycomb repressive complex 2 (PRC2; *EZH1*, *EZH2*, *SIN3A*, *EED*), which catalyzes the methylation of H3K27me3 [52]. As shown in Figure **2a**, no relevant changes in gene expression were detected, neither due to compressive force nor due to fatty acid exposure. We further analyzed the level of H3K27 trimethylation in dual stimulated HPdLF with quantitative immunofluorescence (**Fig. 2b, c**). While baseline levels were comparable, additional application of compressive force led to reduced levels of H3K27me3 under fatty acid and control conditions. However, compared to a 82.68% ± 3.66 reduction in BSA controls, changes in PA cultures (62.17% ± 4.24) were significantly lower (p-value 0.0276 x 10^{-2}, ***).

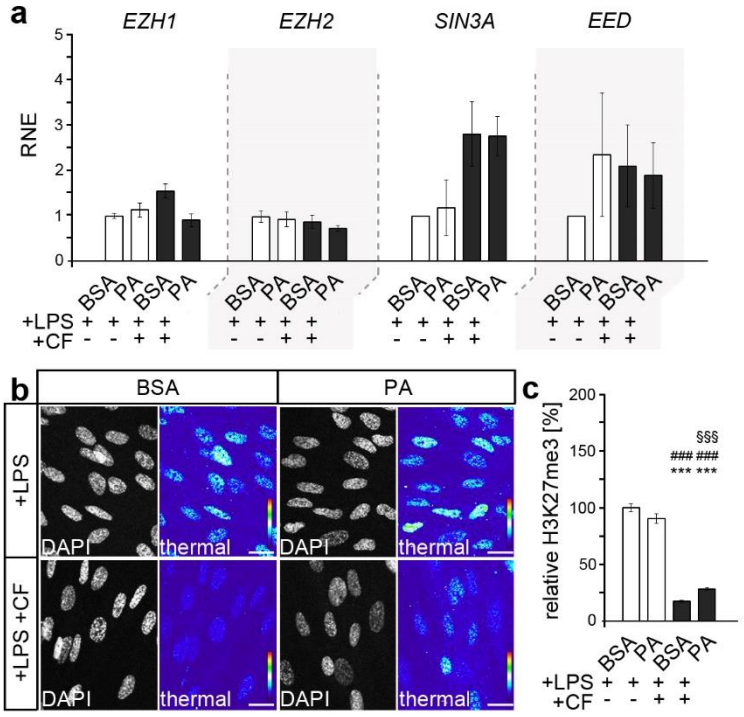

Figure 2: Decrease in H3K27 trimethylation in response to dual stimulation is less in PA-stimulated HPdLFs. (**a**) Quantitative expression analysis of genes encoding components of the polycomp repressive complex (*EZH1*, *EZH2*, *SIN3A*, and *EED*) in human periodontal ligament fibroblasts (HPdLF) exposed to palmitic acid (PA) and simultaneous stimulated with compressive force (CF) and *P. gingivalis* LPS as compared to BSA controls. (**b**, **c**) Representative microphotographs of global H3K27me3 (H3K27me3) in HPdLF treated with PA and co-stimulated with CF and LPS in comparison to BSA controls. Cell nuclei are labeled with DAPI and H3Kac staining intensity is shown in thermal LUT (thermal) analyzed in (c) in relation to BSA control. *** $p < 0.001$ in relation to BSA+LPS, ### $p < 0.001$ in relation to PA+LPS; §§§ $p < 0.001$ in relation to BSA+LPS+CF. Scale bars: 10 µm in (b). RNE, relative normalized expression.

3.2. Inhibition of PRC2 enzymes EZH1 and EZH2 abrogates PA-induced excessive inflammation, possibly via COX2/PGE2 modulation

To evaluate whether these changes in H3K27me3 level are relevant for the pro-inflammatory effects of PA in dual stimulated HPdLF, we inhibited the core enzymes of the PRC2 EZH1 and EZH2 with UNC1999. To avoid a potential toxicity, we first examined the metabolic activity of HPdLFs treated for one day with different concentrations of UNC1999 (**Fig. 3a**). This resulted in a slight positive effect of UNC1999; with the highest effect at a concentration of 1.00 µM.

The activity of H3K27-related histone methyltransferases (HMT) was reduced to 23.23% ± 2.56 in HPdLF treated for one-day with 1.00 µM UNC1999, while lower inhibitor concentrations also resulted in a robust inhibition of HMT activity (**Fig. 3b**). However, for further analysis, we decided to use 1.00 µM UNC1999, which was within the typical range used in previously published *in vitro* studies [53,54].

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To illustrate the inflammatory processes, we performed a THP1 adhesion assay and analyzed the number of activated THP monocytic cells on stimulated HPdLF (**Fig. 3c, d**). Comparable to previous results [44], PA exposure led to an enhanced inflammatory response of dual-stimulated HPdLF as compared to BSA controls, even in the presence of DMSO. While the by UNC1999-reduced activity of EZH1 and EZH2 did not alter the number of adherent THP1 cells in the dual-stimulated BSA control, it reduced the excessive activation of monocytic cells in PA cultures to a level comparable to BSA controls.

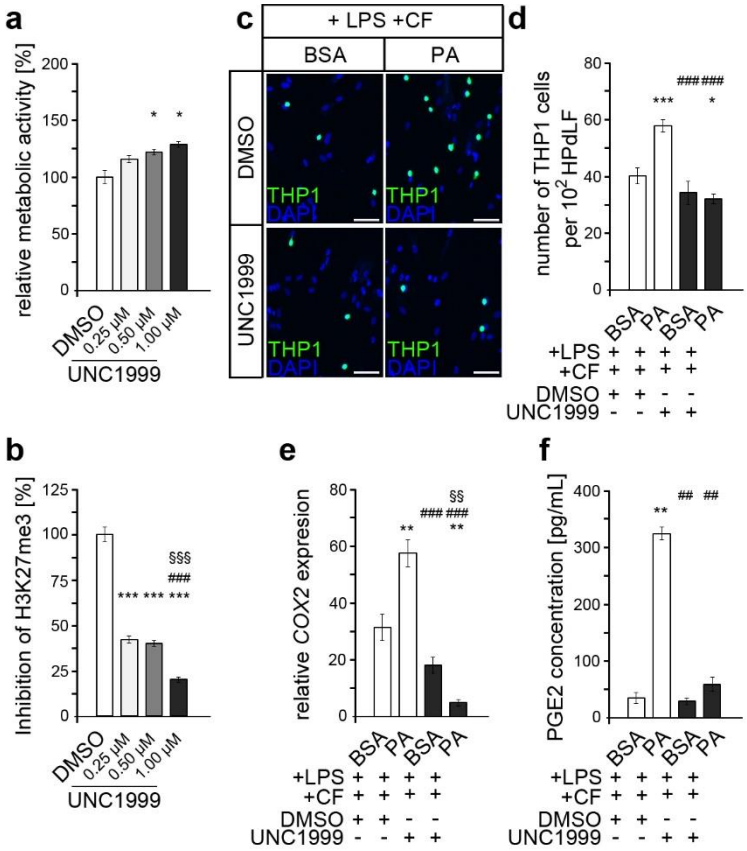

Figure 3: Inhibition of H3K27 trimethylation abrogates the PA-induced excessive inflammatory response of dual stimulated HPdLF. (**a**) Metabolic activity analyzed in human periodontal ligament fibroblasts (HPdLF) treated with different concentrations of UNC1999 for one day in comparison to DMSO. (**b**) Analysis of the activity of histone methyltransferases specifically methylating H3K27 in HPdLF treated with 1.00 µM UNC1999 in comparison to DMSO control. (**c**, **d**) Microphotographs of adherent THP1 monocytic cells (green) on compressed (compressive force, CF) and *P. gingivalis* LPS-stimulated HPdLFs exposed to palmitic acid (PA) after treatment with 1.00 µM UNC1999 in relation to BSA and DMSO controls (c). Cell nuclei were labeled with DAPI (blue). The number of THP1 cells per 102 HPdLF is displayed in (d). (**e**, **f**) Analysis of *COX2* expression levels (e) and PGE2 secretion (f) in dual stimulated HPdLF exposed to PA after treatment with 1.00 µM UNC1999 in comparison to BSA and DMSO controls. * $p < 0.05$ in relation to DMSO in (a) and BSA+LPS+CF+DMSO in (d), ** $p < 0.01$ in relation to BSA+LPS+CF+DMSO in (e) and (f), *** $p < 0.001$ in relation to DMSO in (b) and BSA+LPS+CF+DMSO in (d); ## $p < 0.01$ in relation to PA+LPS+CF+DMSO; ### $p < 0.001$ in relation to DMSO in (b) and PA+LPS+CF+DMSO in (e); §§ $p < 0.01$ in relation to BSA+LPS+CF+UNC1999. Scale bars: 10 µm in (c).

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To further investigate the effects of UNC1999 on inflammatory processes, we determined expression of *COX2* (**Fig. 3e**), a gene encoding an important cytokine that appears to be relevant to the PA-induced excessive inflammatory response of dual stimulated HPdLF [44]. Under DMSO control condition, *COX2* expression levels were significantly higher in PA cultures in comparison to BSA controls. This up-regulated *COX2* transcription was inhibited by application of UNC1999. To confirm a potential impact of altered COX2-levels on related cytokine secretion, we analyzed PGE2 levels in the supernatant of dual stimulated HPdLFs that were additionally treated with UNC1999 (**Fig. 3f**). The detected results supported the previously suggested assumption in the context of *COX2* expression, as the excessive secretion of PGE2 in dual-stimulated PA cultures was attenuated by UNC1999.

Together, these data suggest that altered reduction of H3K27me3 in dual-stimulated PA cultures may contribute to an excessive inflammatory response of HPdLF, possibly via COX2/PGE2 regulation.

3.2. Palmitic acid causes enhanced trimethylation of H3K27 at Il10 promoter-associated regions in dual stimulated HPdLFs

Because H3K27me3 represses nearby genes, a COX2-associated repressor appeared to be increasingly repressed via that epigenetic modification in dual-stimulated PA cultures. In this context, we next investigated the expression of the anti-inflammatory cytokine IL10 (**Fig. 4a**), which regulates *COX2* transcription [55] and whose expression can be affected by fatty acid-dependent changes in histone modification [51]. We detected a significantly lower *IL10* level in dual stimulated PA cultures as compared to BSA controls supporting our hypothesis. Further analysis of the association of trimethylated H3K27 at *IL10* promoter regions via chromatin-immunoprecipitation followed by quantitative PCR revealed increased levels of associated H3K27me3 at two *IL10* promoter-associated sides in dual-stressed HPdLF exposed to PA (**Fig. 4b, c**).

In conclusion, enhanced association with trimethylated H3K27 may condition the decreased expression of *IL10*, which may cause enhanced COX2/PGE signaling in PA exposed HPdLF concomitantly stimulated with *P. gingivalis*-LPS and compressive force.

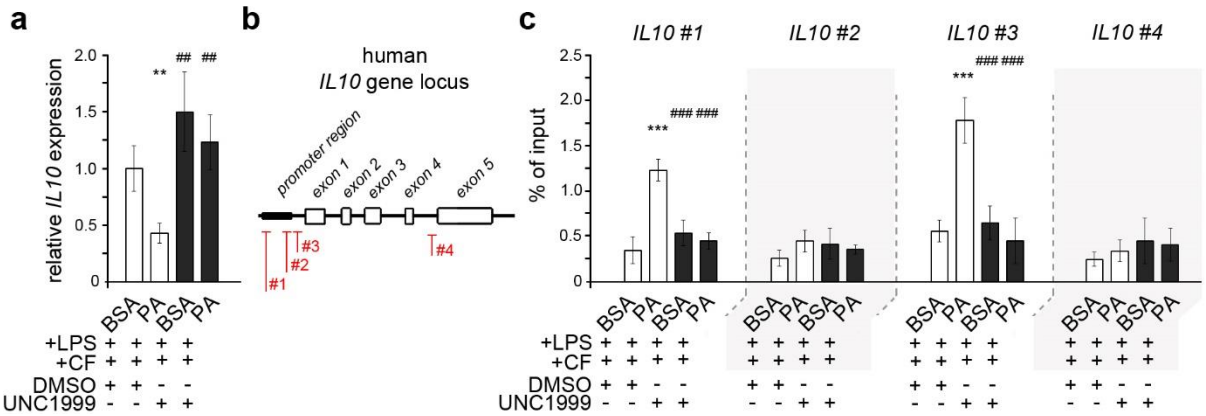

Figure 4: Palmitic acid exposure of dual stressed HPdLF resulted in decreased H3K27 trimethylation near *IL10* promoter regions associated with decreased *IL10* expression. (**a**) Quantitative expression analysis of *IL10* in human periodontal ligament fibroblasts (HPdLF) exposed to palmitic acid (PA) and simultaneous stimulated with compressive force (CF) and *P. gingivalis*-LPS after treatment with 1.00 µM UNC1999 compared to BSA and DMSO controls. (**b**) DNA primer locations in the *IL10* gene locus indicating promoter-associated pairs (#1, #2 and #3) and a non-promoter-associated pair (#4). (**c**) Quantitative analysis of the association H3K27me3 to specific *IL10* gene regions shown in (e) with in dual stimulated HPdLF exposed to PA after UNC1999 treatment in comparison to the respective controls. Data were normalized to the sample input controls. ** $p < 0.01$ in relation to BSA+LPS+CF+DMSO, *** $p < 0.001$ in relation to BSA+LPS+CF+DMSO; ## $p < 0.01$ in relation to PA+LPS+CF+DMSO; ### $p < 0.001$ in relation to PA+LPS+CF+DMSO.

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4. Discussion

This study investigated the impact of palmitic acid-induced epigenetic changes on the hyper-inflammatory response of HPdLFs that had been simultaneously stimulated with *P. gingivalis*-LPS and compressive force. While the force-induced increase in global H3K acetylation was not affected by PA in LPS-stimulated HPdLFs, reduction of global H3K27 trimethylation was less pronounced in PA cultures. Inhibitor studies suggested an enhanced EZH1/EZH2 activity in dual stimulated PA-exposed HPdLFs as cause for enhanced COX2/PGE2 signaling. Since EZH1/EZH2-modulated H3K27me3 is a repressive mark, increased association at the *IL10* promoter provides a possible molecular mechanism via diminished IL10-associated *COX2* down-regulation for excessive inflammation in dual stimulated PA cultures.

Epigenetic alterations of pro-inflammatory cytokines are well described for both periodontitis and obesity [5,56]. Further, mechanical forces were reported to alter histone marks as well, thus mediating force-dependent gene expression [51,57]. It is therefore likely that epigenetic transcriptional control represents a common level at which mechanical and bacterial influences, as well as obesity-related hyperlipidemic effects, may interfere.

We found force-induced changes in global H3K acetylation and H3K27 trimethylation in LPS-stimulated HPdLF, which, however, were only different for repressive H3K27me3 through PA exposure. This seems surprising, since in compressed HPdLF cultured with monounsaturated oleic acid an increase in global histone acetylation had been detected [51]. However, in this recent study, fibroblasts were not additionally stimulated by *P. gingivalis*-LPS, which has been shown to increase HAT expression in human periodontal ligament stem cells (HPdLSC) [58] and, therefore, minor palmitic acid-induced changes in histone acetylation processes may have been override. This might be due to different pathways that convert lipids to acetyl-CoA, which is necessary for histone acetylation [21,40]. While long-chain fatty acids such as PA need to be actively transported across the mitochondrial membrane where acetyl-CoA conversion takes place [59], shorter fatty acids including pathogenic lipid metabolites could diffuse across those membranes, leading to rapid oxidation to acetyl-CoA [60]. However, it is also known that different fatty acids can have varying influences on histone acylation by regulating distinct enzymes [61].

In accordance with other studies [62,63], we detected reduced global H3K27 trimethylation in HPdLF that were challenged by *P. gingivalis*-LPS and compressive force. However, at least at the RNA level, we could not detect altered expression of the major H3K27-HMT EZH1 and EZH2, which other studies have shown in force-stressed HPdLSC and pathogenically stimulated human B cell lymphoma cells BCBL1 [62,63]. Moreover, we detected increased global H3K27me3 due to PA exposure in dual stressed HPdLF, which is in contradiction to other studies reporting reduced levels of this histone mark [42] or enhanced expression of associated histone demethylases (HMD) such as JMJD3 [64] when stimulated with this fatty acid. This could be due to a variety of reasons including the different cell types and the used PA concentrations as well as the combination of several stimuli possibly leading to different changes in HMT or HMD activity. In this context, posttranslational protein modifications such as acetylation, methylation, phosphorylation, ubiquitination, and O-GlcNAcylation, which can be influenced by various environmental factors [65], were shown to affect the stability and nuclear localization of components of the PRC2 complex as well as its HMT activity and binding of other proteins [66]. Further studies could focus on PA-related changes in the activity of the PRC2 complex to further elucidate the mechanistic background for the increased H3K27me3 level in dual stimulated HPdLF, but this was beyond the scope of our study.

Previously, we reported that PA exposure excessively promoted COX2/PGE2 signaling in *P. gingivalis* LPS stimulated HPdLF that were simultaneously stressed for six hours by compressive force resulting in enhanced activation of monocytic THP1 cells [44]. Similar to our previous study, we detected increased PGE2 secretion in dual-stimulated HPdLF, even when they were additionally cultured with DMSO as an inhibitor control.

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This is consistent with the literature describing increased PGE2 levels in various cells upon both compression and *P. gingivalis* stimulation in addition to fatty acid exposure [16,67-72]. In this study, we now observed a correlatively increased expression of *COX2* in dual-stressed HPdLF after PA exposure. This should be taken with caution, as it could also be triggered by DMSO eliciting pro- and anti-inflammatory effects in a cell type-, concentration-, and duration-dependent manner [73]. Nevertheless, both excessive *COX2* expression and PGE2 secretion induced by PA exposure in dual-stimulated HPdLF were counterbalanced by UNC1999, which promotes lower H3K27 trimethylation [74]. Transcriptional repression of *COX2* is related to high levels of H3K27me3 associated with the gene promoter region, which when removed by EZH2-specific inhibition with DZNep led to increased expression [75]. However, also other epigenetic modifications such as histone acetylation and DNA methylation modulating COX2 transcription highlight a complex regulatory network of this important inflammatory signaling mediator [75,76]. Moreover, we used UNC1999, which also inhibits EZH1 in a concentration-dependent manner [74]. Since EZH1 has similar functions but not fully overlapping targets with EZH2, potential differences could arise from additional inhibition of EZH1. This is also supported by the study of Yamagishi *et al.*, who reported different clusters of regulated genes for both HMTs with a more important role of EZH1 in the inflammatory response [77]. This may also explain why UNC1999 attenuates the excessive activation of THP1 cells to control levels in dual stimulated HPdLF exposed to PA. Future studies may elucidate the specific roles of both H3K27-HMTs in this complex regulation of the *COX2*/PGE2-driven inflammatory response.

Our study provides evidence that epigenetically modulated *IL10*-related *COX2* regulation is altered in PA-exposed HPdLFs that were concomitantly stressed by mechanical and bacterial stimuli; potentially resulting in this hyper-inflammatory response. The negative regulatory effect of the anti-inflammatory cytokine IL10 on *COX2* expression is described comprehensively [55]. Comparable to our results, palmitic acid stimulation of adipocytes resulted in diminished *IL10*-expression associated with reduced protein secretion [78] further promoting the pro-inflammatory effect of this fatty acid. We detected an enhanced association of trimethylation H3K27 at two position in or close to the *IL10* promoter region of dual-stressed HPdLF exposed to PA. Transcriptional repression of *IL10* by H3K27 trimethylation is also reported for other cell types, including T helper cells (Th)0, Th2 and iTreg cells [79] as well as Th17 [80]. In this regard, EZH2 knockdown resulted in increased levels of IL10 [79], which we also observed for gene expression due to pharmacological inhibition with UNC1999. Based on our data, it can be speculated that IL10 is inhibited by a PA-mediated increase in H3K27me3 and, therefore, may fail to limit the COX2/PGE2-driven inflammatory response in dual-stimulated HPdLFs.

Our study is limited for several reasons, including the use of a single fatty acid and LPS as a single pathogenic stimuli, each at one specific concentration, and the study of a single duration of compressive force. Even though the ratio of palmitic acid to BSA has been reported for serum levels of obese patients, *in vivo* the ratio to other fatty acids are also decisive for hyperlipidemia-related health problems [81-85]. This makes further clinical studies indispensable. After six hours of compressive force one could detect an early phase of inflammatory response, however COX2/PGE2 signaling was reported to be already increased [86,87]. More critical is the remarkable complexity of epigenetic regulation. In this context, changes in one modification require adaptations of other modifications. It has been shown that a reduction of H3K27 trimethylation can lead to increased global and gene-specific levels of activating modifications such as H3K4me3 and H3Kac [88]. However, elucidating these changes in detail was beyond the scope of this study, which aimed to establish a first potential link between the hyper-inflammatory response of dual-stressed hyperlipidemic PdL fibroblasts and the epigenetic regulation of potential key factors. Because of a potential reversibility of this obesity-related adaptation of the inflammatory response, H3K27me3 inhibitors may be of therapeutic interest in addition to the HDAC inhibitors that have already been explored [89]. This is of particular interest considering the increasing frequency of obese patients requiring orthodontic treatment

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and the relevance of periodontal disease in view of associated risks such as tooth root resorption and tooth loss. In the treatment of periodontitis, there are already therapeutic approaches involving modulation of the inflammatory response of the host cells [90]. Epigenetic remodeling may provide a novel platform to overcome unfavorable disease-induced cellular changes by treatment with epigenetic inhibitors during tooth movement.

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