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ORIGINAL ARTICLE

Regulation of visfatin by microbial and biomechanical signals in PDL cells

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Abstract
Objectives This in vitro study was established to examine whether visfatin thought to be a link between periodontitis and obesity is produced by periodontal ligament (PDL) cells and, if so, whether its synthesis is modulated by microbial and/or biomechanical signals.
Materials and methods PDL cells seeded on BioFlex® plates were exposed to the oral pathogen *Fusobacterium nucleatum* ATCC 25586 and/or subjected to biomechanical strain for up to 3 days. Gene expression of visfatin and toll-like receptors (TLR) 2 and 4 was analyzed by RT-PCR, visfatin protein synthesis by ELISA and immunocytochemistry, and NFκB nuclear translocation by immunofluorescence.
Results F. nucleatum upregulated the visfatin expression in a dose- and time-dependent fashion. Preincubation with neutralizing antibodies against TLR2 and TLR4 caused a significant inhibition of the *F. nucleatum*-upregulated visfatin expression at 1 day. *F. nucleatum* stimulated the NFκB nuclear translocation. Biomechanical loading reduced the stimulatory effects of *F. nucleatum* on visfatin expression at 1 and 3 days and also abrogated the *F. nucleatum*-induced NFκB nuclear translocation at 60 min. Biomechanical loading inhibited significantly the expression of TLR2 and TLR4 at 3 days. The regulatory effects of *F. nucleatum* and/or biomechanical loading on visfatin expression were also observed at protein level.
Conclusions PDL cells produce visfatin, and this production is enhanced by *F. nucleatum*. Biomechanical loading seems to be protective against the effects of *F. nucleatum* on visfatin expression.
Clinical relevance Visfatin produced by periodontal tissues could play a major role in the pathogenesis of periodontitis and the interactions with obesity and other systemic diseases.

Keywords Biomechanics · Tension · Forces · *Fusobacterium nucleatum* · Visfatin · Periodontal ligament

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Introduction

Periodontitis is a chronic inflammatory disease caused by oral pathogenic microorganisms present in dental plaque. These periodontopathogens as well as their components and

metabolic products can elicit local immunoinflammatory reactions in periodontal tissues. As a consequence of the exaggerated inflammatory processes and immune responses, periodontal soft and hard tissues are subjected to degradation and resorption, respectively, which can result in periodontal attachment and even tooth loss [1, 2]. Moreover, periodontitis has been shown to be associated with cardiovascular diseases, diabetes mellitus, rheumatoid arthritis, obesity, metabolic syndrome, and other systemic diseases and conditions [3].

The periodontal tissues are periodically exposed to biomechanical loading during mastication, speech, and dental habits. Since the periodontal ligament (PDL) represents a richly vascular and cellular connective tissue, it can absorb and distribute physiological forces, which are at the same time critical for remodeling and maintenance of the periodontium [4, 5]. However, dental overloading due to occlusal discrepancies, dental habits, or strong orthodontic forces can be harmful to periodontal tissues and contribute to progressive periodontal destruction [6–8]. It is well known from in vitro and clinical studies, that biomechanical loading can induce the synthesis of inflammatory mediators and proteases, thereby acting as proinflammatory and catabolic signals [9, 10]. For example, excessive mechanical stress due to hyperocclusion has been demonstrated to stimulate osteoclastogenesis and alveolar bone destruction [11]. In summary, both bacterial infection and biomechanical loading can cause immunoinflammatory reactions and, thereby, destructive processes in the periodontium. However, if and how microbial infection and biomechanical loading interact on the level of periodontal cells has yet to be elucidated.

Recently, an association between periodontal diseases and obesity has been reported [12]. Systemic low-grade inflammation present in obesity may be a pathomechanistic link between obesity and other chronic diseases, such as periodontitis [13]. In the adipose tissue, a number of cytokines, such as visfatin, leptin, resistin, and adiponectin, are produced and released [14]. These adipokines not only regulate insulin sensitivity and energy expenditure, but also proinflammatory and wound healing processes. Whereas adiponectin exerts anti-inflammatory effects, visfatin, leptin, and resistin possess proinflammatory characteristics [15, 16]. Interestingly, the serum levels of these adipokines are altered in obesity and overweight [15, 16]. It is thought that adipokines not only contribute to the subclinical inflammatory state in obesity, but that such molecules also represent the pathomechanistic link between type 2 diabetes, obesity, and periodontitis [17].

Visfatin is predominantly secreted by cells from human visceral adipose tissue and was originally denominated as pre-B cell colony enhancing factor. This protein has also an enzymatic nicotinamide phosphoribosyltransferase activity responsible for the synthesis of nicotinamide adenine dinucleotide which is essential for cell metabolism. Visfatin induces the synthesis of proinflammatory cytokines and acts as a chemotactic factor [18–20]. High serum levels of visfatin have been found in a number of inflammatory diseases and conditions, such as obesity, type 2 diabetes, metabolic syndrome, atherosclerosis, cancer, rheumatoid arthritis, and sepsis [21–27]. Recently, it has been demonstrated that visfatin is present in high levels in gingival crevicular fluid (GCF) and serum from periodontally diseased patients, indicating that visfatin might also be produced in the periodontium and regulated by bacterial infection and/or inflammation [28–30]. However, it is as yet unknown whether PDL cells produce this proinflammatory adipokine, and if so, whether its synthesis is regulated by oral bacteria and biomechanical loading. Hence, the aim of this study was to examine in vitro whether visfatin is produced in PDL cells and, if so, whether its synthesis is modulated by microbial and/or biomechanical signals.

Materials and methods

Culture and treatment of cells

PDL cells from periodontally healthy donors, who underwent tooth extraction for orthodontic reasons, were used. Written informed parental consent and approval of the Ethics Committee of the University of Bonn were obtained. Cells from nine donors were collected from the middle third of the tooth roots and cultured in Dulbecco's Modified Eagle's Medium (Invitrogen, Karlsruhe, Germany) supplemented with 10 % fetal bovine serum (FBS, Invitrogen), 100 units of penicillin and 100 μg/ml of streptomycin (both from Biochrom, Berlin, Germany) at 37 °C in a humidified atmosphere of 5 % CO_2. Cells of passages 3–5 were seeded (1×10^5 cells/well) on six-well BioFlex® collagen-coated culture plates (Flexcell International, Hillsborough, NC, USA) and grown to 80 % confluence. One day prior to experiments, the FBS concentration was reduced to 1 %. The medium was changed every other day during the course of the experiment. In order to mimic bacterial infection in vitro, cells were stimulated with the inactivated oral pathogen *Fusobacterium nucleatum* ATCC 25586 (optical density 0.025, 0.05, and 0.1). The strain was precultivated 48 h on Schaedler agar plates (Oxoid, Basingstoke, UK) in an anaerobic atmosphere. Thereafter, bacteria were suspended in phosphate-buffered saline (PBS) ($OD_{660nm}=1$, equivalent to 1.2×10^9 bacterial cells/ml) and exposed two times to ultrasonication (160 W for 15 min) resulting in a complete killing. Moreover, biomechanical loading conditions were simulated in vitro by application of cyclic tensile strain (CTS) of low (CTSL, 3 %) and high (CTSH, 20 %) magnitudes at a rate of 0.05 Hz to cells. Like in previous

experiments, a strain device (CESTRA) developed at the University of Bonn was used to apply biomechanical loading [31–34]. In the present study, cells were exposed to *F. nucleatum* ATCC 25586, CTS, and their combination for 1 and 3 days, respectively.

Real-time RT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following manufacturer's protocol. By using the iScript™ Select cDNA Synthesis Kit (Bio-Rad, Munich, Germany), 500 μg of total RNA was reverse transcribed at 42 °C for 90 min followed by 85 °C for 5 min, following manufacturer's instructions. Gene expression of visfatin, toll-like receptor (TLR) 2 and 4, and glyceraldehyde-3-phosphate dehydrogenase as housekeeping gene was analyzed by real-time RT-PCR using the iCycler iQ detection system (Bio-Rad), SYBR Green (Qiagen), and specific primers (QuantiTect Primer Assay, Qiagen). One microliter of cDNA was amplified as a template in a 25-μl reaction mixture containing 12.5 μl of 2x QuantiFast SYBR Green PCR Master Mix (Qiagen), 2.5 μl of primers and RNase free water. The mixture was heated initially at 95 °C for 5 min and then followed by 50 cycles of denaturation at 95 °C for 10 s and combined annealing/extension at 60 °C for 30 s. Data were analyzed using the comparative threshold cycle method.

ELISA

Protein levels of visfatin in cell supernatants were measured using a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The absorbance was determined using a microtiter plate reader (POWERWAVE X; BioTek Instruments, Winooski, VT, USA) at 450 nm. Data were normalized by the cell number.

Immunofluorescence

Cells attached to the flexible bottom of the culture plates were fixed with 4 % paraformaldehyde in PBS pH7.4 (Sigma-Aldrich, Munich, Germany) for 10 min at room temperature (RT), washed with PBS, and treated with 0.1 % Triton X-100 (Sigma-Aldrich) for 5 min at RT. Then cells were washed again with PBS and blocked with a blocking buffer (nonfat dry milk; Bio-Rad) for 1 h at RT. After washing, the cells were incubated with primary rabbit antibody NFκB p65 (1:400; Cell Signaling Technology, Danvers, MA, USA) for 90 min and with secondary antibody CY3 (1:2,000; Abcam, Cambridge, MA, USA) for 45 min. Cells were observed under a×20 objective using an Axioplan 2 imaging microscope (Carl Zeiss MicroImaging, Jena, Germany). The images were captured with a PVCAM camera and the VisiView capturing software (Visitron Systems, Puchheim, Germany).

Immunocytochemistry

Cells were cultured on glass coverslips in a 24-well plate. Cells were stimulated or not with *F. nucleatum* ATCC 25586 and stained for the presence of visfatin. After 3 days, cells were fixed in 4 % paraformaldehyde at pH7.4 and RT for 15 min and then permeabilized in 0.1 % Triton X-100 for 5 min. Nonspecific antigens were blocked by incubation with serum block (LSAB System; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 20 min. Subsequently, cells were incubated overnight at 4 °C with rabbit polyclonal antibody to visfatin (Santa Cruz Biotechnology). Afterwards, cells were labeled with goat anti-rabbit IgG-HRP secondary antibody (Cell Signaling Technology) for 45 min. For staining, cells were exposed to DAB chromogen (Dako, Hamburg, Germany) for 3 min at RT. After each incubation step, cells were washed twice with PBS. Counterstaining was performed with Mayer's Hematoxylin (Merck Eurolab, Dietikon, Switzerland) for 2 min. Coverslips were mounted in DePex mounting medium (Serva Electrophoresis, Heidelberg, Germany). Standardized photomicrographs were taken using an Axioplan 2 imaging microscope (Carl Zeiss MicroImaging).

Statistical analysis

Statistical analysis of the data was performed using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). For quantitative analysis, values were expressed as mean and standard errors of the mean. One-way analysis of variance followed by the post hoc Dunnett's or Tukey's tests was applied. Differences between groups were considered significant at $p<0.05$. All experiments were performed in triplicate and repeated at least twice.

Results

Time- and dose-dependent stimulation of visfatin production by *F. nucleatum* ATCC 25586

First, we sought to examine whether PDL cells produce visfatin and, if so, whether this production is regulated by bacteria. Our experiments revealed that visfatin was constitutively expressed by PDL cells and upregulated by *F. nucleatum* ATCC 25586 (Fig. 1a,b). As shown in Fig. 1a, *F. nucleatum* ATCC 25586 had no significant effect on visfatin mRNA expression up to 12 h, but it stimulated significantly the visfatin expression at 24 h (3.8-fold; $p<$

0.05) and 72 h (3.9-fold; $p<0.05$). Furthermore, the *F. nucleatum*-induced increase in visfatin expression was dose-dependent. At 24 h, low concentration of *F. nucleatum* ATCC 25586 enhanced the visfatin expression by 3.8-fold ($p<0.05$), whereas higher concentrations caused a more than 6-fold increase in its expression ($p<0.05$) (Fig. 1b). The stimulatory effect of *F. nucleatum* ATCC 25586 on visfatin was also observed at protein level by immunocytochemistry and ELISA (Fig. 1c and Table 1).

Regulation of *F. nucleatum*-induced effects on visfatin by biomechanical loading

Next, we studied whether the response of PDL cells to *F. nucleatum* ATCC 25586 is modulated by biomechanical loading, because the periodontium is often subjected simultaneously to microbial infection and biomechanical, i.e., occlusal, loading. Biomechanical loading alone slightly decreased the constitutive visfatin mRNA expression in PDL cells at 1 and 3 days, but these effects were not significant (Fig. 1d and e). However, biomechanical loading modulated the actions of *F. nucleatum* ATCC 25586 on visfatin in PDL cells. CTSL abrogated the stimulatory effect of *F. nucleatum* ATCC 25586 on visfatin by 17 % ($p>0.05$) and by 70 % ($p<0.05$) at 1 and 3 days, respectively. CTSH reduced the *F. nucleatum*-induced stimulation of visfatin expression by 86 % ($p<0.05$) at 1 day and completely blocked ($p<0.05$) the visfatin upregulation at 3 days (Fig. 1d,e). The counter-regulatory effects of CTSL and CTSH were also observed at protein level, as analyzed by ELISA (Table 1).

Involvement of TLRs in the actions of *F. nucleatum* ATCC 25586 on visfatin

We then sought to unravel the mechanisms underlying the stimulatory effect of *F. nucleatum* ATCC 25586 on visfatin expression. PDL cells were preincubated with neutralizing antibodies against TLR2 and TLR4 and, subsequently, stimulated with *F. nucleatum* ATCC 25586. Our experiments revealed that preincubation with antibodies against TLR2 and TLR4 caused a significant ($p<0.05$) inhibition of the *F. nucleatum*-induced visfatin stimulation by 20 and by 24 %, respectively, at 1 day (Fig. 2a).

Exploitation of the NFkB pathway by *F. nucleatum* ATCC 25586

Upon ligand binding, TLRs can trigger the NFκB signaling pathway. We therefore examined whether *F. nucleatum* ATCC 25586 activates this pathway in PDL cells. As

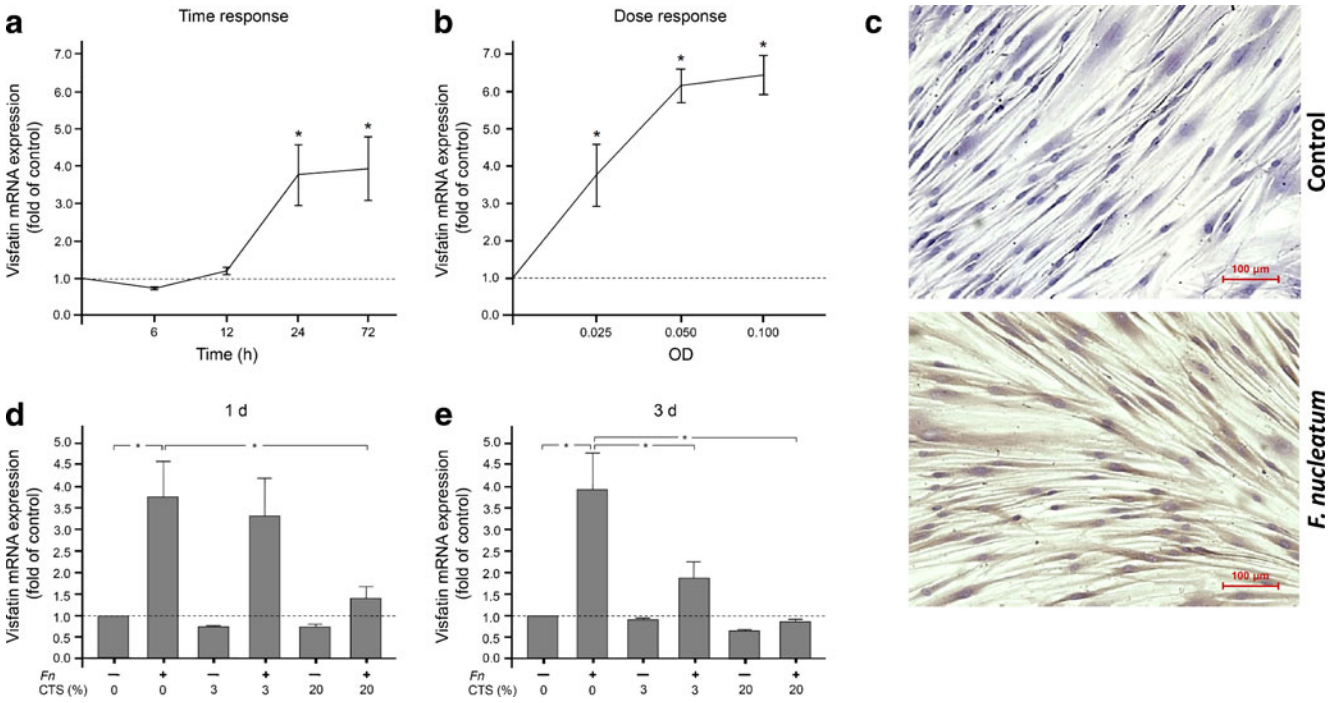

Fig. 1 **a** Stimulation of visfatin expression by *F. nucleatum* ATCC 25586 (OD, 0.025) in PDL cells over time. *: significantly ($p<0.05$) different from unstimulated cells ($n=9$); **b** Stimulation of visfatin expression by various concentrations of *F. nucleatum* ATCC 25586 in PDL cells at 24 h. *: significantly ($p<0.05$) different from unstimulated cells ($n=9$); **c** Visfatin protein synthesis in PDL cells in the presence and absence of *F. nucleatum* ATCC 25586 (OD, 0.025) at 3 days, as analyzed by immunocytochemistry. Images from one representative experiment are shown; **d** and **e** Regulation of visfatin expression in the presence of *F. nucleatum* ATCC 25586 (*Fn*; OD, 0.025) and/or biomechanical loading (CTS) of low (3 %) and high (20 %) magnitudes in PDL cells at 1 (**d**) and 3 days (**e**). *: significant ($p<0.05$) difference between groups ($n=9$)

Table 1 Visfatin protein levels in supernatants of PDL cells treated with *F. nucleatum* ATCC 25586 either alone or in combination with low (CTSL) and high (CTSH) biomechanical loading

Groups	Visfatin protein synthesis (ng/10^5 cells)	
	Day 1	Day 3
Control	213.44±1.10	143.34±1.68
F. nucleatum	1030.22±9.40*	354.83±3.22*
F. nucleatum + CTSL	725.28±6.50**	253.05±2.34**
F. nucleatum + CTSH	613.35±5.30**	132.18±1.39**

* $p<0.05$ (significantly different from control); ** $p<0.05$ (significantly different from control and *F. nucleatum*-stimulated cells)

evidenced by immunofluorescence microscopy, *F. nucleatum* ATCC 25586 stimulated the nuclear translocation of NFκB and caused a maximal NFκB accumulation within the nucleus at 60 and 90 min (Fig. 2b). Then, we examined whether this *F. nucleatum*-stimulated NFκB transactivation is regulated by biomechanical loading. As demonstrated in Fig. 2c, CTSL and CTSH remarkably inhibited the *F. nucleatum*-induced NFκB nuclear translocation at 60 min.

Regulation of TLRs by biomechanical loading

We then examined whether the inhibitory effects of biomechanical loading on *F. nucleatum* ATCC 25586 actions might be caused by downregulation of TLRs. Although CTSL had no regulatory effect on TLR2 at 1 day, CTSH reduced significantly the constitutive TLR2 mRNA expression by 35 % ($p<0.05$) at this time point (Fig. 2d). At 3 days, the TLR2 expression was significantly inhibited by 12 % ($p<0.05$) by CTSL and by 69 % ($p<0.05$) by CTSH (Fig. 2d). The TLR4 expression was not significantly affected by biomechanical loading at 1 day (Fig. 2e). At 3 days, the expression of TLR4 was inhibited by 12 % ($p<0.05$) by CTSL and by 52 % ($p<0.05$) by CTSH (Fig. 2e).

Discussion

This study shows for the first time that the proinflammatory adipokine visfatin is produced by PDL cells. In addition, visfatin was upregulated by the oral pathogen *F. nucleatum* ATCC 25586, and this increase was significantly counterregulated by biomechanical loading. These findings suggest that visfatin may be produced at sites of periodontal infection with *F. nucleatum* ATCC 25586 and, thereby, contributes to periodontal inflammation and destruction. By contrast, biomechanical loading of the PDL might be at least partially protective against the stimulatory effects of *F. nucleatum* ATCC 25586 on visfatin.

Recently, the role of visfatin in inflammatory diseases and conditions has been widely investigated. Plasma visfatin levels have been found to be increased in patients with obesity, metabolic syndrome, diabetes mellitus, cardiovascular diseases, and insulin resistance [21–27]. Furthermore, it has also been demonstrated that body-weight reduction by physical exercise can lead to decreased visfatin levels [35]. In recent years, evidence has accumulated that obese individuals have an increased risk of periodontitis, and it is thought that adipokines might represent the pathomechanistic link between both diseases [12, 17]. Visfatin has been shown to stimulate a variety of cells to produce inflammatory mediators and proteases by using different intracellular pathways [36]. Moreover, this adipokine can inhibit apoptosis of inflammatory cells [37, 38]. However, if and how visfatin affects periodontal cells is as yet unclear. Interestingly, Pradeep and coworkers have recently reported increased visfatin levels in GCF from gingivitis and periodontitis patients, as compared to periodontally healthy individuals [28–30]. Furthermore, periodontal treatment caused a decrease in visfatin levels [28–30]. These reports suggest that visfatin might also be produced in the periodontium and that its synthesis is enhanced by periodontal infection. Our study demonstrates for the first time that PDL cells produce visfatin and, thereby, suggests that PDL cells may contribute to the increased visfatin levels in GCF in periodontitis. However, if and to what extent other immunoinflammatory or structural cells of the periodontium produce visfatin has yet to be elucidated.

In the present study, *F. nucleatum* ATCC 25586, which was used to mimic a bacterial infection, increased the visfatin production in a dose- and time-dependent manner. *F. nucleatum* is a gram-negative, anaerobic microorganism, acts as a bridge bacterium between early and late colonizers during plaque development, and is associated with both gingivitis and periodontitis [39, 40]. In vitro studies have shown that *F. nucleatum* ATCC 25586 can invade epithelial cells, fibroblasts, and PDL cells [41–43]. In addition, *F. nucleatum* ATCC 25586 supports other periodontal pathogens to invade host cells [44]. Nevertheless, periodontitis is caused by a complex bacterial biofilm. Further studies should clarify whether other microorganisms associated with periodontitis are also capable of stimulating the synthesis of visfatin.

Although biomechanical loading alone had only minor effects on the visfatin production in PDL cells, they inhibited the *F. nucleatum*-induced upregulation of visfatin in a magnitude-dependent manner. These findings indicate that biomechanical loading, for example, caused by occlusal loading or orthodontic treatment may not necessarily augment the stimulatory action of *F. nucleatum* ATCC 25586 on visfatin. At least for the strain regimens used in our experiments, biomechanical loading had a protective role

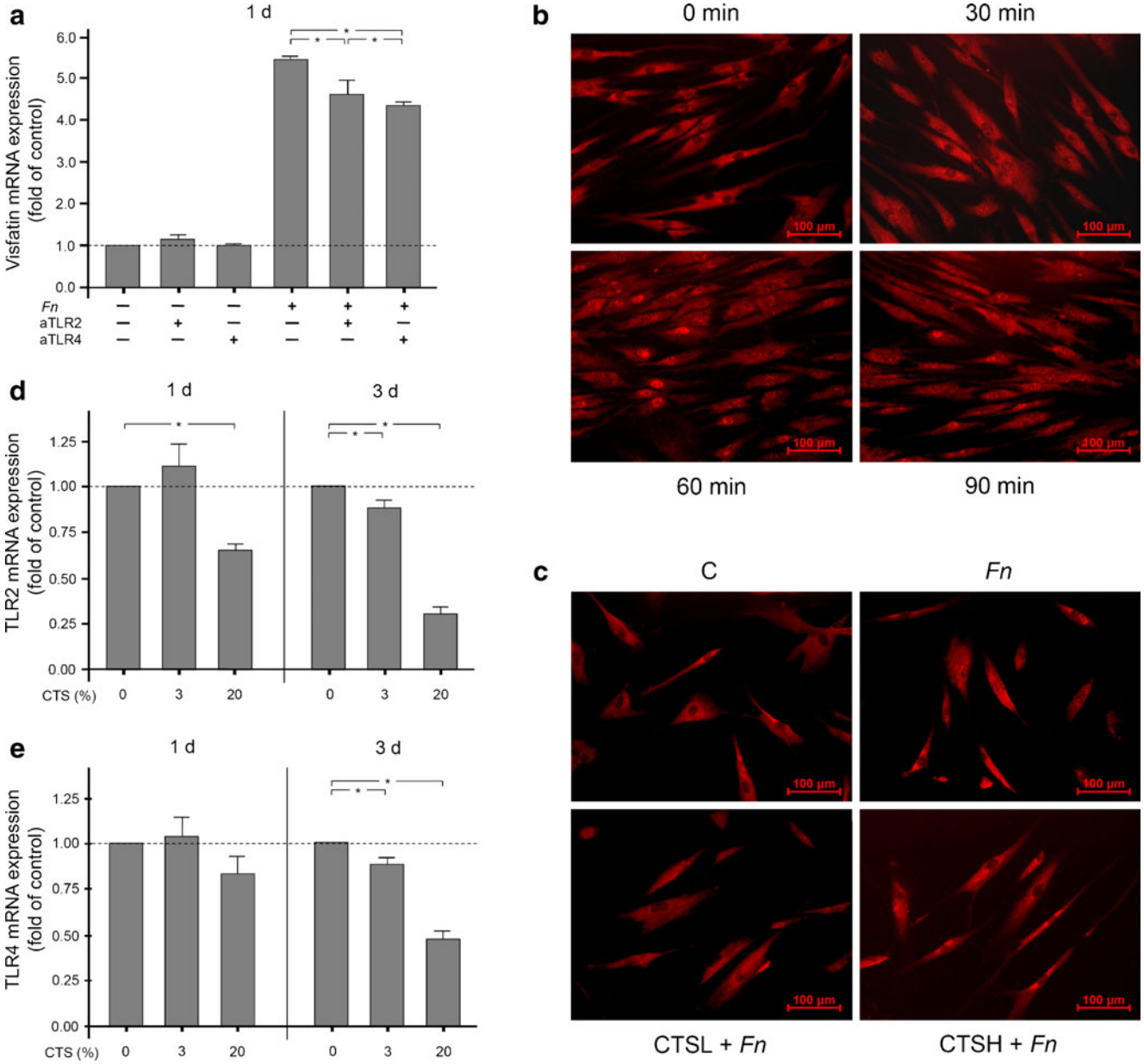

Fig. 2 **a** Stimulation of visfatin expression by *F. nucleatum* ATCC 25586 (OD, 0.025) in the presence and absence of blocking TLR2 or TRL4 antibodies in PDL cells at 1 day. Unstimulated PDL cells in the presence and absence of blocking antibodies served as controls. *: significant ($p<0.05$) difference between groups ($n=9$). **b** Stimulation of NFκB nuclear translocation by *F. nucleatum* ATCC 25586 (OD, 0.025) over time, as analyzed by immunofluorescence. Images from one representative experiment are shown; **c** Inhibition of *F. nucleatum* (*Fn*; OD, 0.025)-stimulated p65 nuclear translocation by biomechanical loading of low (CTSL; 3 %) and high (CTSH; 20 %) magnitudes in PDL cells at 60 min. Images from one representative experiment are shown; **d** and **e** Expression of TLR2 (**d**) and TLR4 (**e**) in PDL cells subjected to biomechanical loading of low (3 %) and high (20 %) magnitudes at 1 and 3 days. *: significant ($p<0.05$) difference between groups ($n=9$)

against the actions of *F. nucleatum* ATCC 25586 on this proinflammatory adipokine. Our results concur with previous reports, which have also demonstrated that biomechanical loading can exert anti-inflammatory effects [31, 45, 46].

Our study revealed that one of the pathways by which *F. nucleatum* ATCC 25586 upregulates visfatin are TLR2 and TLR4. Interestingly, biomechanical loading decreased time- and magnitude-dependently the expression of these TLRs, suggesting that the counterregulatory effects of CTS on the action of *F. nucleatum* ATCC 25586 might be mediated at least in part by downregulation of these receptors, especially at 3 days. TLRs are strong activators of the NFκB pathway [47]. *F. nucleatum* LPS was shown to be a strong ligand for TLR2 and a weak ligand for TLR4 for activation of NFκB [48]. Interestingly, we could observe that CTS inhibited the NFκB nuclear translocation stimulated by *F. nucleatum*

ATCC 25586, indicating that both *F. nucleatum* ATCC 25586 and CTS exploit this pathway for their opposite effects.

In our experiments, a suspension of *F. nucleatum* ATCC 25586 was used. Since this suspension was exposed to intensive ultrasonication, it can be assumed that the suspension contained disrupted cell wall particles with a high amount of LPS. However, other bacterial components also present in the suspension may interact with the receptors. The *F. nucleatum* ATCC 25586 concentration used in this study was determined by dose–response experiments. A low *F. nucleatum* concentration, as can be found in subgingival plaque, was chosen for the subsequent experiments, because this concentration exerted a pronounced stimulatory effect on visfatin, while also allowing to study the modulatory effects of biomechanical loading on the *F. nucleatum*-stimulated visfatin synthesis [49].

Like in previous experiments, tensile forces were applied to the cells [31–34]. However, during mastication, dental habits, and orthodontic treatment, the tooth-supporting periodontal tissues are subjected to complex forces. Whether compressive, hydrostatic, and shear forces as well as their combinations exert similar effects, as observed for tensile strain in the present study, needs to be examined. As in our previous investigations, cells were exposed to biomechanical loading of low and high magnitudes, which have been shown to occur in the periodontium [50, 51].

In summary, the present study demonstrates for the first time that visfatin is produced by PDL cells. Moreover, the oral pathogen *F. nucleatum* ATCC 25586 caused an upregulation of visfatin, whereas biomechanical loading counterregulated the *F. nucleatum*-induced stimulation of this adipokine. Within the limits of this study, we conclude that visfatin may be produced by the PDL in the presence of periodontal infection with *F. nucleatum* ATCC 25586. In addition, biomechanical loading of the PDL might be at least partially protective against the *F. nucleatum*-induced stimulation of visfatin synthesis.

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Conflict of interest The authors declare that they have no conflict of interest.

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