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ORIGINAL ARTICLE

Inflammatory cytokines are suppressed by light-emitting diode irradiation of *P. gingivalis* LPS-treated human gingival fibroblasts

Inflammatory cytokine changes by LED irradiation

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Abstract Human gingival fibroblasts (hGFs) play an important role in the inflammatory reaction to lipopolysaccharide (LPS) from *P. gingivalis*, which infects periodontal connective tissue. In addition, although light-emitting diode (LED) irradiation has been reported to have biostimulatory effects, including anti-inflammatory activity, the pathological mechanisms of these effects are unclear. This study examined the effects of 635-nm irradiation of *P. gingivalis* LPS-treated human gingival fibroblasts on inflammatory cytokine profiles and the mitogen-activated protein kinase (MAPK) pathway, which is involved in cytokine production. Gingival fibroblasts treated or not treated with *P. gingivalis* LPS were irradiated with 635-nm LED light, and cytokine profiles in the supernatant were assessed using a human inflammation antibody array. Expression of cyclooxyginase-2 (COX-2) protein and phosphorylation of extracellular signal-regulated kinase (ERK 1/2), p38, and c-Jun-N-terminal kinase (JNK) were assessed by Western-blot analysis to determine the effects on the MAPK pathway, and prostaglandin E_2 (PGE_2) in the supernatant was measured using an enzyme-linked immunoassay. COX-2 protein expression and PGE_2 production were significantly increased in the LPS-treated group and decreased by LED irradiation. LPS treatment of gingival fibroblasts led to the increased release of the pro-inflammatory-related cytokines interleukin-6 (IL-6) and IL-8, whereas LED irradiation inhibited their release. Analysis of MAPK signal transduction revealed a considerable decrease in p38 phosphorylation in response to 635-nm radiation either in the presence or absence of LPS. In addition, 635-nm LED irradiation significantly promoted JNK phosphorylation in the presence of LPS. LED irradiation can inhibit activation of pro-inflammatory cytokines, mediate the MAPK signaling pathway, and may be clinically useful as an anti-inflammatory tool.

Keywords Light emitting diode irradiation · Inflammation · Cytokine · PGE_2

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Introduction

Porphyromonas gingivalis (*P. gingivalis*) has been implicated as a major etiological agent in the development and progression of periodontal disease [1, 2]. Inflammation following *P. gingivalis* infection leads to the destruction of periodontal tissue and resorption of alveolar bone, which can ultimately result in tooth loss [3]. A likely candidate for the initiation of a destructive inflammatory response to *P. gingivalis* is lipopolysaccharide (LPS). This bacterial cell wall component is a potent stimulator of the innate host defense system [4]. Periodontal disease progression is regulated by the host response to *P. gingivalis*, and there is increasing evidence that non-immune cells, such as human gingival fibroblasts (hGFs), participate in the host response [5]. hGFs respond to *Escherichia coli* (*E. coli*) and *P. gingivalis* LPS by producing inflammatory cytokines, such as interleukin-6 (IL-6) and IL-8 [6]. Other studies have reported that hGFs express Toll-like receptors and CD14, which is consistent with the finding that these cells respond to LPS [7, 8]. These results have also shown that *P. gingivalis* infection up-regulates the expression of a range of chemokines, cytokines, and other pro-inflammatory factors, mediated by complex intracellular signal transduction through modulated MAPK pathways, such as extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal protein kinase (JNK), and p38 MAPK.

Several recent reports have suggested that visible light irradiation is an effective alternative treatment that reduces inflammation caused by periodontal disease [9–11]. Many physiological studies have reported that low-level laser treatment (LLLT) and light emitting diode treatment (LEDT) can reduce pain and inflammation and accelerate wound healing [12]. Some studies have also suggested that irradiation at a wavelength of 635 nm decreases intracellular reactive oxygen species (ROS) and mitigates oxidative stress [13–16]. It has also been reported that 635-nm irradiation decreases inflammation by inhibiting PGE_2 production and COX-1 and -2 mRNA expression in in vitro and in vivo models [12, 17]. Although previous studies have reported that LLLT can improve inflammation, it is unclear whether cytokines are activated by irradiation in the inflammatory state.

This study investigated cytokine profiles in response to 635-nm irradiation of *P. gingivalis* LPS-treated hGFs. Given that hGFs show an inflammatory response to *P. gingivalis* infection, the major aim of this study was to reveal the activation profiles of inflammatory cytokines in response to 635-nm irradiation [18]. In addition, we investigated signal transduction involved in cytokine production using a phosphorylation assay for ERK, JNK, and p38 MAPK because these MAPK pathway components are involved in the pro-inflammatory process after infection with *P. gingivalis*.

Materials and methods

Cell culture and chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human gingival fibroblasts (hGFs) were obtained from a healthy 34-year-old male visiting Chonnam National University Hospital. The gingival tissues were cut finely with scissors and cultured in alpha minimum essential medium (a-MEM) (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (Biomeda Co., Hayward, CA, USA) and a 1% antibiotic–antimycotic solution (Welgene, Daegu, Korea) at 37°C in a 5% CO_2 humidified chamber. The medium was replaced with fresh medium, and adherent hGF cells were allowed to reach approximately 70% confluence. The cells were detached using trypsin-ethylenediamine tetraacetic acid (trypsin-EDTA: Gibco BRL) solution and re-plated (subcultured) at a density of 1×10^5/ml in 10-cm plates for each experiment. The plates were divided randomly into four treatment groups: Control (Con), 635-nm irradiated (Ir), *P. gingivalis* lipopolysaccharide (InvivoGen, San Diego, CA, USA) treated (LPS), and *P. gingivalis* LPS treated with 635-nm irradiation (LPS + Ir).

Light source and irradiation

The light source for irradiation was a continuous-wave light emitting diode (LED, U-JIN LEDs Co., Korea) with a wavelength of 635 nm and an power density of 5 mW/cm^2 (Biophoton Co., Korea). LED irradiation was performed for 1 h [14].

Western-blot analysis

Twenty-four hours after irradiation, the medium was removed and the cells were washed twice with PBS. Cell lysates were then prepared in 200 μl of cold lysis buffer (1% NP-40, 50 mM Tris-HCL, pH 7.5, 150 mM NaCl, 0.02% sodium azide, 150 μg/ml PMSF, 2 μg/ml aprotinin, 20 μg/ml leupeptin and 1 μg/ml pepstatin A). Thirty milligrams of cell lysate was separated on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Amersham, USA). The membrane was blocked with a blocking solution (5% skim milk in TBST (2.42 g/l Tris-HCL, 8 g/l NaCl, 0. % Tween 20, pH 7.6)) for 30 min

and rinsed briefly in TBST. The membrane was incubated overnight at 4°C with anti-COX-2 antibody (1:1,000), anti-phospho-JNK antibody (1:500, Cell Signaling, Beverly, MA, USA), anti-JNK antibody (1:1,000, Cell Signaling), anti-phospho-p38 antibody (1:500, Cell Signaling), anti-p38 antibody (1:1,000, Cell Signaling), anti-phospho-ERK1/2 antibody (1:500, Cell Signaling), anti-ERK1/2 antibody (1:1,000, Cell Signaling), and anti-GAPDH antibody (1:3,000) in 5% non-fat milk. After rinsing with TBST, the membrane was incubated for 1 h with anti-rabbit and anti-mouse horseradish peroxidase-conjugated (1:2,000) secondary antibody. Finally, the membrane was washed in TBST and the immunoreactivity of the proteins was detected using an enhanced chemiluminescence (ECL) detection kit.

Enzyme-linked immunosorbent assay for PGE_2

After hGFs were incubated in the presence or absence of LPS for 24 h, PGE_2 was measured in the supernatant using a commercially available enzyme immunosorbent assay kit (R&D System, Minneapolis, MN, USA) according to the manufacturer's protocol. The absorbance of PGE_2 was measured at 450 nm using a colorimetric microplate reader (Biotek, Winooski, VT, USA).

Cytokine profiling

To assess cytokine production profiles, the supernatant from cultured gingival fibroblasts was collected and assayed using a human inflammation antibody array (R&D Systems) according to the instruction manual. This method uses a membrane coated with specific antibodies for each cytokine forming an array. Table 1 lists the cytokines studied using this technique. The cytokine signal was detected using an ECL detection kit (Amersham, USA) and quantified by densitometric analysis using Scion Image software (Scion Corp, MD, USA). The signal intensity of each spot was adjusted to the corresponding internal control provided for each membrane by the manufacturer (cytokine profiling). The ratio of expression was calculated by dividing the signal intensity of each cytokine by the signal intensity of the internal control for each sample.

Statistical analysis

All experiments were carried out in triplicate. Data are expressed as means ± standard deviations. Statistical differences were determined by analysis of variance (ANOVA) using SPSS for Windows version 12.0 (SPSS, USA). Statistical significance was set at $p<0.05$.

Results

The effects of 635-nm irradiation on LPS-treated hGFs were assessed by measuring COX-2 expression and PGE_2 production by Western-blot analysis and an enzyme-linked immunoassay, respectively. COX-2 protein expression was increased significantly in the LPS-treated group and was decreased in the LPS + Ir group (Fig. 1a, b).

LPS treatment in hGFs also increased PGE_2 production to 1.4-fold the level of controls, whereas irradiation led to significantly lower PGE_2 levels in the LPS + Ir group relative to LPS-treated cells (Fig. 1c).

To assess cytokine production, supernatants from hGFs cultured in the presence or absence of LPS were collected after 24 h. Cytokine profiles were assessed using a cytokine antibody array that can detect 40 different cytokines. Supernatants of non-stimulated cells contained basal levels of some cytokines, particularly IL-6 and IL-8. Cytokines were classified into four groups (Table 1) and the results are presented accordingly.

Cytokines were assessed using three innate cytokines and five chemokines. Growth-regulated oncogene-alpha (GRO alpha), interleukin-6 (IL-6), and macrophage migration inhibitory factor (MIF) of innate cytokines were measured in the media (Fig. 2). Among these, IL-6 showed the greatest induction in response to LPS, increasing significantly to 1.3-fold of the control level (Fig. 2b). However, 635-nm irradiation led to a decrease in IL-6 release, reducing levels to those of control cells.

Among the chemokines, interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), serpin E1, regulated on activated normal T-cell expressed and secreted (RANTES), and stromal cell-derived factor-1 (SDF-1) were assayed (Fig. 3). IL-8, an important mediator of the innate immune response, was increased significantly to 1.3-fold the level of controls in LPS-treated hGFs (Fig. 3a). In the LPS + Ir group, IL-8 was significantly decreased to the level of controls. Only a slight difference was observed with radiation alone. RANTES was diminished to 0.6-fold of unchallenged controls after LPS treatment. In contrast, it was elevated significantly to 0.8-fold of control levels in the LPS + Ir group (Fig. 3d). Only a slight decrease in SDF-1 was observed in the LPS + Ir group.

We next examined the effects of LPS and 635-nm irradiation on MAPK activity by measuring phosphorylation of extracellular signal-regulated kinase (ERK1/2) (Fig. 4b), p38 (Fig. 4c), and c-Jun-N-terminal kinase (JNK) (Fig. 4d) in hGFs (Fig. 4). LPS treatment led to a slight increase in ERK1/2 phosphorylation, but no obvious changes in ERK1/2 phosphorylation were detected after 635-nm irradiation. However, a consider-

Table 1 List of human inflammatory cytokines examined using the antibody array (R&D Systems)

Coordinate	Target/control	Alternate nomenclature	Name	Cytokine group
A1, A2	Positive control	–	–	–
A2, A3	C5a	Complement component 5a	–	–
A5, A6	CD40 Ligand	CD154	–	–
A7, A8	G-CSF	CSFβ, CSF-3	Granulocyte colony-stimulating factor	Growth factors
A9, A10	GM-CSF	CSFα, CSF-2	Granulocyte -macrophage colony-stimulating factor	Growth factors
A11, A12	GROα	CXCL1	Growth regulated oncogene-alpha	Innate
A13, A14	I-309	CCL1	Immune-associated nucleotide	Chemokine
A15, A16	sICAM-1	CD54	–	–
A17, A18	IFN-τ	Type II IFN	Interferon-τ	Acquired
A19, A20	Positive control	–	–	–
B3, B4	IL-1α	IL-1F1	Interleukin-1α	Innate
B5, B6	IL-1β	IL-1F2	Interleukin-1β	Innate
B7, B8	IL-1ra	IL-1F3	–	Innate
B9, B10	IL-2	–	Interleukin-2	Acquired
B11, B12	IL-4	–	Interleukin-4	Acquired
B13, B14	IL-5	–	Interleukin-5	Innate
B15, B16	IL-6	–	Interleukin-6	Innate
B17, B18	IL-8	CXCL8	Interleukin-8	Chemokine
C3, C4	IL-10	–	Interleukin-10	Acquired
C5, C6	IL-12 p70	–	Interleukin-12 subunit 70	Innate
C7, C8	IL-13	–	Interleukin-13	Acquired
C9, C10	IL-16	LCF	Interleukin-16	Chemokine
C11, C12	IL-17	–	Interleukin-17	Growth factors
C13, C14	IL-17E	–	Interleukin-17E	–
C15, C16	IL-23	–	Interleukin-23	–
C17, C18	IL-27	–	Interleukin-27	–
D3, D4	IL-32α	–	Interleukin-32α	–
D5, D6	IP-10	CXCL10	Interferon-inducible protein	Chemokine
D7, D8	I-TAC	CXCL11	–	Chemokine
D9, D10	MCP-1	CCL2	Monocyte chemoattractant protein-1	Chemokine
D11, D12	MIF	GIF, DER6	Macrophage migration inhibitory factor	Innate
D13, D14	MIP-1α	CCL3	Macrophage inflammatory protein-1α	Chemokine
D15, D16	MIP-1β	CCL4	Macrophage inflammatory protein-1β	Chemokine
D17, D18	Serpin E1	PAI-1	–	Chemokine
E1, E2	Positive control	–	–	–
E3, E4	RANTES	CCL5	Regulated on activated normal T-cell expressed and secreted	Chemokine
E5, E6	SDF-1	CXCL12	Stromal cell-derived factor-1	Innate
E7, E8	TNF-α	TNFSF1A	Tumor necrosis factor-α	Innate
E9, E10	sTREM-1	–	–	–
E19, E20	Negative control	–	–	–

able decrease in the level of P38 phosphorylation was observed after 635-nm irradiation both in the presence and absence of LPS. In addition, irradiation promoted JNK phosphorylation in the presence of LPS. These results suggest that the effects of 635-nm irradiation on the MAPK signaling pathway are primarily mediated by P38 and JNK phosphorylation.

Discussion

Periodontal disease comprises a group of infections that can lead to gingival inflammation, periodontal tissue destruction, and tooth and alveolar bone loss in severe cases [8, 19]. The host response is initiated by recognition of LPS, which is present in the Gram-negative bacterium *P. gingivalis*. The

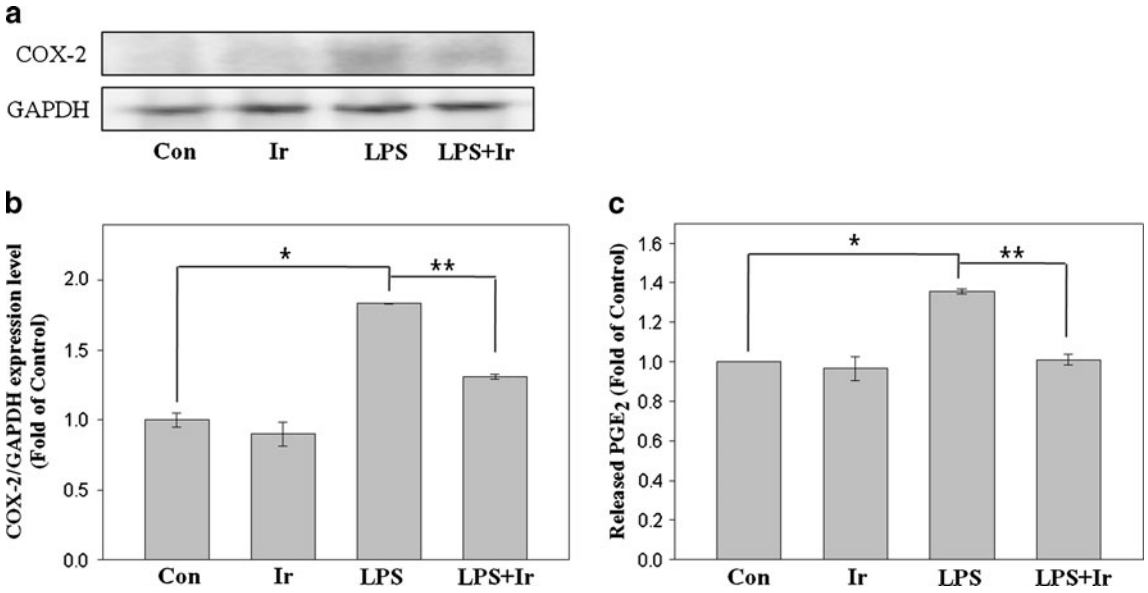

Fig. 1 Effects of 635-nm irradiation on *P. gingivalis* lipopolysaccharide (LPS)-induced cyclooxygenase-2 (COX-2) expression and PGE_2 production in hGFs. These experiments were performed with or without 635-nm light irradiation and in the presence or absence of LPS (10 μg/ml) for 24 h. **a** Cells were prepared for immunodetection with anticyclooxygenase-2 antibodies. The membrane was denuded and GAPDH was used to normalize protein content. **b** Densitometric analysis of COX-2 represents the mean ratios ± SD from three separate experiments. Significant differences are indicated as $*p < 0.05$ compared to control and $**p < 0.05$ compared to LPS. **c** PGE_2 was measured by ELISA of culture medium from the previous experiment

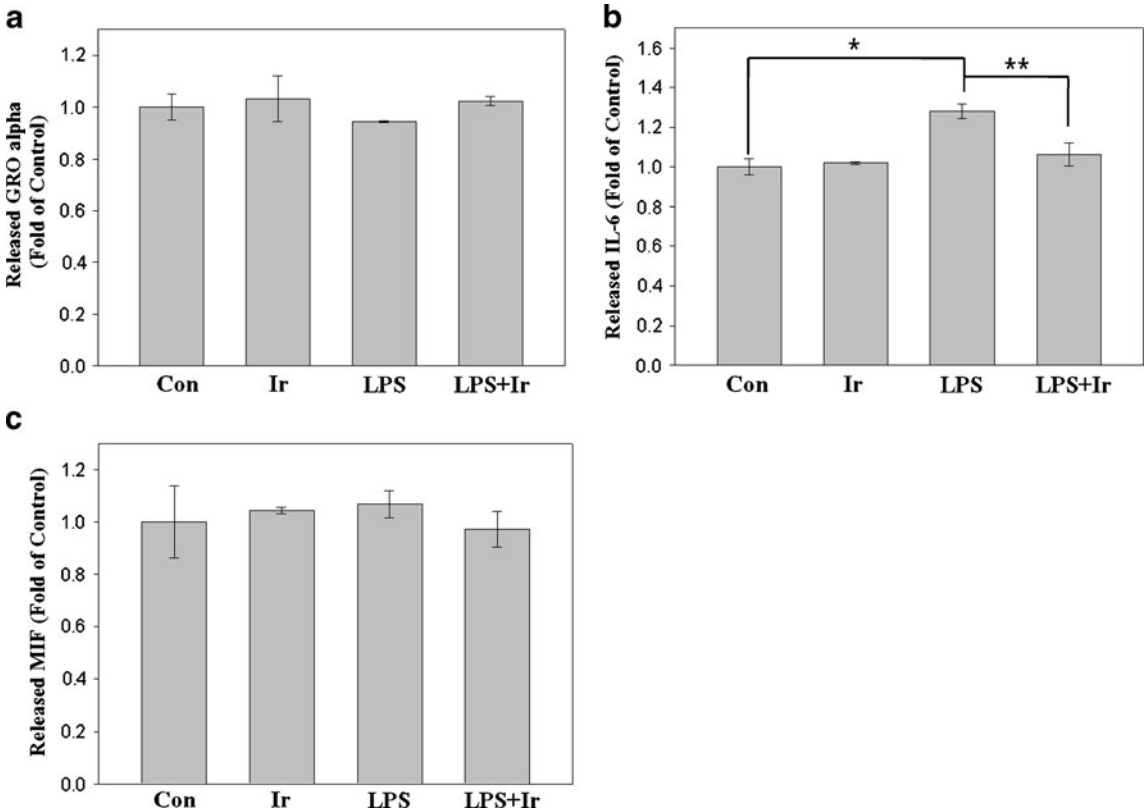

Fig. 2 Effects of 635-nm irradiation on innate cytokine production after 24 h LPS treatment. The supernatant of LPS-treated hGFs was harvested after 24 h and assayed using a cytokine profile array kit. **a** GRO alpha, **b** IL-6, and **c** MIF content in the supernatant are shown. *Bars* denote the mean ratios ± SD from two experiments. Significant differences are indicated as $*p < 0.05$ compared to control and $**p < 0.05$ compared to LPS

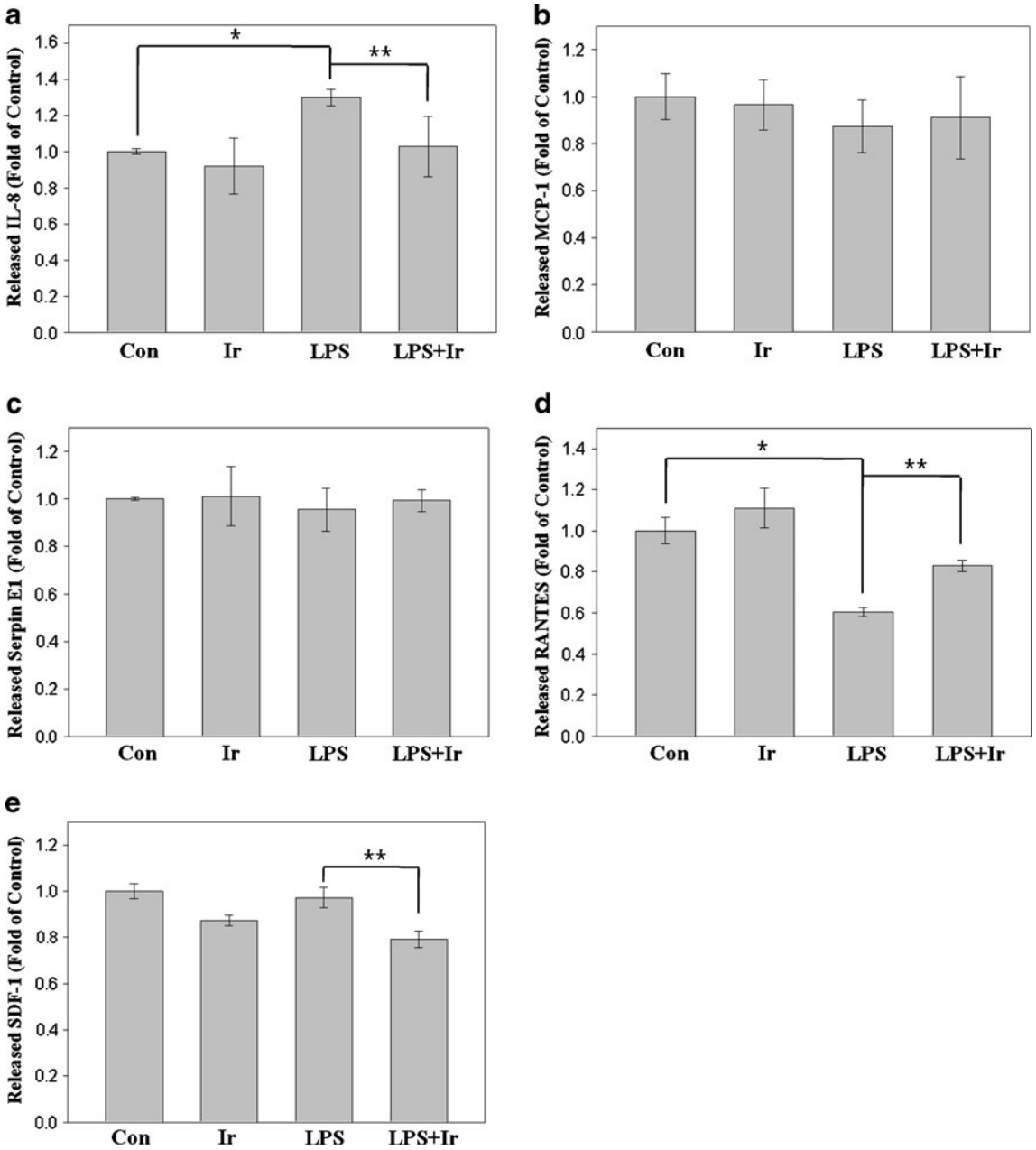

Fig. 3 Effects of 635-nm irradiation on chemokine production after 24-h LPS treatment. **a** IL-8 release, **b** MCP-1, **c** Serpin E1, **d** RANTES, and **e** SDF-1 are shown. *Bars* represent the mean ratios ± SD from two experiments. Significant differences are indicated as $*p < 0.05$ compared to control and $**p < 0.05$ compared to LPS

external membranes of these bacteria contain LPS, which interacts with Toll receptors, activating transduction pathways and culminating in inflammatory cytokine expression. IL-1 and PGE_2 are among the cytokines involved in periodontal disease pathogenesis and are present at high concentrations in the crevicular fluid and periodontal tissues of diseased sites.

Recently, low-level laser therapy (LLLT) has gained considerable recognition for its wound healing and anti-inflammatory properties [20, 21]. It has been reported that 635-nm irradiation can reduce inflammation as effectively as indomethacin or ibuprofen, which are established anti-inflammatory agents [12]. In the present study, human gingival fibroblasts were treated with *P. gingivalis* LPS to induce inflammation. To confirm the presence of inflammation, PGE_2 and COX-2 protein expression was measured. The ability of 635-nm light irradiation to reduce inflammation was tested, and we found that PGE_2 and

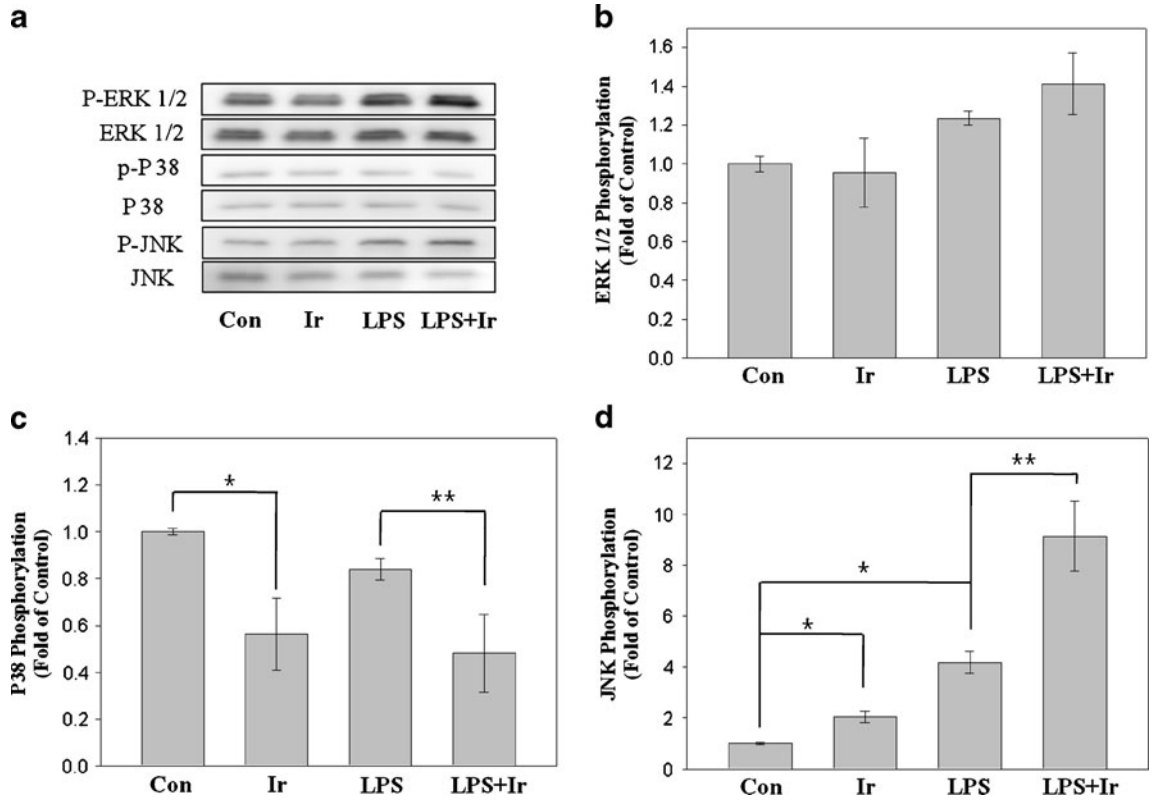

Fig. 4 Effects of 635-nm irradiation on phosphorylation of extracellular signal-regulated kinase ½ (ERK 1/2), p38, and Jun-N-terminal kinase (JNK) induced by LPS in hGFs. **a** Cells were processed and analyzed by Western blot using anti-phospho ERK 1/2, anti-phospho p38, and anti-phospho JNK. The membranes were denuded and antibodies against non-phosphorylated ERK 1/2, p38, and JNK were used to control for total protein content. The results obtained were similar in three separate experiments; the mean ratio ± SD was obtained by densitometry, as shown in the graphic analysis. **b** ERK 1/2, **c** p38, and **d** JNK are shown. Significant differences are indicated as $*p < 0.05$ compared to control and $**p < 0.05$ compared to LPS

COX-2 expression were reduced by 635-nm irradiation. In a previous study, 635-nm irradiation was shown to dissociate intracellular ROS, which mediate $cPLA_2$, $sPLA_2$, and COX-2 expression, and inhibit the release of PGE_2.

Gingival fibroblasts can produce innate pro-inflammatory cytokines and chemokines. Overproduction of potent osteoclast activators, pro-inflammatory cytokines, and chemokines within the periodontal connective tissues establishes a perfect microenvironment for periodontal destruction [18]. Cytokines and chemokines are involved in the initiation and effector stages of immunity and inflammation and regulate the amplitude and duration of the response [5]. They are generally produced transiently, are extremely potent, typically act at picomolar concentrations, and interact with specific cell-surface receptors normally expressed at relatively low levels [22]. Irradiation at 635 nm significantly decreased IL-6 and IL-8 production in *P. gingivalis* LPS-treated hGFS. IL-6 and IL-8 are "secondary" pro-inflammatory cytokines that act in an autocrine manner. Their function is mediated by "primary" cytokines such as IL-1b, which is secreted within minutes in response to various stimuli [23]. This may increase vascular permeability to IL-6 and enhance the recruitment of leukocytes to the region, including those expressing IL-8 [24]. The fact that IL-6 and IL-8 were observed at higher levels in LPS-treated hGFs indicates that primary cytokines were effective in immediately enhancing the inflammatory response, and that IL-6 and IL-8 release is elevated in an autocrine manner. However, the decreased IL-6 and IL-8 levels seen in hGFs treated with LPS and irradiation resulted from autocrine suppression of the inflammatory response.

RANTES, also known as CCL5, is a chemokine produced in response to stimulation by cytokines, such as IL-6 and TNF-α, and is secreted by a variety of cells, including vascular smooth muscle cells (VSMCs), epithelial cells, T cells, macrophages, and platelets [25, 26]. RANTES exerts a multitude of generally pro-inflammatory effects and is of importance in T cell and monocyte chemoattraction, T cell proliferation, and the delayed-type hypersensitivity response. In this study, RANTES was decreased by LPS treatment and elevated significantly in response to LPS and 635-nm irradiation. Some reports have stated that RANTES levels are inversely correlated with disease

severity and mortality in individuals with severe thrombocytopenia [27]. Cytokines are known to play a vital role in the immunopathology of an ever-increasing number of diseases, and appropriate cytokine production is essential for the development of protective immunity [5]. Severe disease can result from an inappropriate cytokine response. It is not clear how the immune system selects the proper response to a particular pathogen. However, determining the features of both the host and pathogen that direct how and where the organism is presented to cytokine-producing cells is essential for a better understanding of the pathogenesis of not only periodontal disease but of all infectious diseases.

To address the question of how 635-nm irradiation stimulates MAPK signal transduction, phosphorylation of ERK1/2, JNK, and P38 was measured in LPS-treated hGFs. Mitogen-activated protein kinases (MAPKs) are serine-threonine kinases that mediate intracellular signaling associated with a range of cellular activities, including cell proliferation, differentiation, survival, death, and transformation [28, 29]. The mammalian MAPK family consists of ERK, p38, and JNK (also known as stress-activated protein kinase or SAPK). MAPK pathways are activated either as a result of a series of binary interactions between kinase components or through formation of a signaling complex containing multiple kinases that are guided by a scaffold protein. Such scaffold proteins mediate activation of MAPK signaling pathways, consisting of specific kinase components. In this study, 635-nm irradiation affected phosphorylation of p38 and JNK. The JNK and p38 signaling pathways are activated by pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, and in response to cellular stresses, such as genotoxic, osmotic, hypoxic, or oxidative stress [8]. Especially, p38 is activated by cytokine (TNF-alpha) or chemokines by an autocrine manner. p38 activation was considered to be inhibited by 635-nm LED irradiation in LPS-treated gingival fibroblast, which increased the synthesis of several proinflammatory mediators such as COX-2, and PGE_2 via transcriptional regulation [30]. Unlike p38, JNK is mainly activated by growth factors (e.g., FGF, EGF, and PDGF etc.) and inflammatory cytokine as well. In numerous reports, LED irradiation was known to accelerate the wound-healing process via the release of various growth factors increase [21]. In the present study, increased growth factors by 635-nm irradiation were considered to stimulate the JNK phosphorylation in the absence or presence of LPS. Thus, 635-nm irradiation can affect MAPK activation and is correlated with the inhibition of pro-inflammatory cytokine expression.

Conclusions

Considering the anti-inflammatory effects of 635-nm irradiation based on the present study, the inflammatory cytokine profile after 635-nm irradiation indicates that 635-nm irradiation relieves cellular stress and decreases COX-2 expression and PGE_2 release in *P. gingivalis* LPS-treated hGFs mediate the MAPK signaling pathway, resulting in a decrease in the release of pro-inflammatory cytokines. This evidence suggests that 635-nm irradiation mitigates inflammation and may be clinically useful as an anti-inflammatory treatment.

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