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Melatonin exerts anti-fibrinolytic effects by regulating IL-1β-induced changes in uPA, uPAR, and PAI-1 expression/production in human dental pulp cells

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Abstract

Interleukin-1β (IL-1β) is a pro-inflammatory cytokine and its expression is increased in inflamed dental pulp. IL-1β affects plasminogen activation system molecules, which are crucial for tissue inflammation, fibrinolysis, matrix turnover, and cell adhesion and migration. Melatonin, which provides circadian and seasonal signals, is a physiological endocrine generated by the pineal gland. It has anti-oxidant and anti-inflammatory properties. Studies are warranted to determine whether melatonin prevents IL-1β-induced expression/production of plasminogen system molecules. Human dental pulp cells (HDPCs) were exposed to IL-1β or melatonin alone or to IL-1β with/without pretreatment with melatonin or other inhibitors. The mRNA expression of uPA, uPAR, and PAI-1 was quantified using real-time polymerase chain reaction analysis. The cellular uPA, PAI-1, and soluble uPAR (suPAR) production was determined using an enzyme-linked immunosorbent assay. Signaling molecules’ protein expression was analyzed by immunofluorescent staining. We found that IL-1β (0.1–10 ng/mL) stimulated uPA and uPAR expression/production but inhibited PAI-1 expression/production of HDPCs. Melatonin inhibited uPA but stimulated uPAR/suPAR and PAI-1 expression/production. Intriguingly, melatonin prevented IL-1β-induced uPA mRNA expression/production. Conversely, melatonin enhanced the IL-1β-induced uPAR and PAI-1 mRNA expression/protein production of HDPCs. IL-1β-induced suPAR production was attenuated by U0126 (a MEK/ERK inhibitor), SB203580 (a p38 inhibitor), and 5Z-7oxozeaenol (a TAK1 inhibitor), whereas SB203580 prevented an IL-1β-induced decline of PAI-1 production. Moreover, melatonin attenuated the IL-1β-induced p-ERK, p-p38, p-Akt and p-TAK1. These results revealed the crucial role of IL-1β in the pathogenesis of pulpal inflammation/repair via stimulation of uPA and uPAR and inhibition of PAI-1, which can be differentially regulated by p38, Akt, MEK/ERK, and TAK1. Melatonin exerts an anti-fibrinolytic effect on IL-1β-induced changes in uPA, uPAR, and PAI-1 in HDPCs. Clinically, the melatonin levels of patients may affect pulpal inflammatory response. Melatonin and signal transduction inhibitors may be administered concomitantly for the prevention and treatment of pulpal inflammatory diseases.

Keywords: Dental pulp, Inflammation, Interleukin-1β, Melatonin, Plasminogen activation system molecules

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1. Introduction

Dental pulp typically suffers from various exogenous insults, such as invasion of microorganisms resulting from dental caries or crown fracture with pulp exposure and irritation from dental restorative materials. Dental pulp has an intrinsic capacity to repair and regenerate lost tissue. However, transient or persistent inflammatory responses of dental pulp may also occur to different extents in many offending teeth to eliminate invading pathogens and allow tissue repair [1]. Identifying the inflammatory, repair, and regeneration processes of dental pulp is therefore crucial for the successful control of pulpal inflammatory diseases and the prevention of pulp necrosis.

Numerous cellular molecules, such as interleukin (IL)-1β, IL-1α, IL-8, urokinase plasminogen activator (uPA), and IL-6, are stimulated during pulpal inflammation to control infection/inflammation and induce the cellular defense system of the host for repair [2–5]. A low level of inflammation may promote tissue repair [1], whereas chronic/persistent pulpal inflammation and cytokine release may lead to irreversible pulpsitis, the impairment of pulpal repair, and eventually pulp necrosis [5]. Increased expression of IL-1β and IL-8 in pulpsitis tissues has been reported in comparison to healthy dental pulp [6]. IL-1β stimulates the mRNA expression and secretion of uPA in Human dental pulp cells (HDPCs) [4]. Similarly, recent studies have reported the stimulation of IL-8, uPA, and ICAM-1 expression and production by IL-1β in HDPCs, and these events are differentially regulated by TAK1, MEK/ERK, p38, and IRAKs [7,8].

Plasminogen activators exist in two forms, uPA and tissue PA (tPA), that may convert plasminogen into its active form, plasmin. PA promotes cell migration and proliferation and is involved in numerous physiological and pathological conditions, such as inflammation and tissue remodeling [9]. TLR5 activation may stimulate uPA in HDPCs [10]. The expression of tPA is elevated in inflamed dental pulp relative to healthy dental pulp [11]. The degradation of primary matrix tissue by uPA plays a critical role in the formation of granulation tissue [12]. The effect of uPA is tightly controlled by uPA receptor (uPAR), soluble uPAR (suPAR), and plasminogen activator inhibitor (PAI-1). Both uPAR and suPAR may mediate the effect of uPA, but with some differences [13]. However, little is known about the effect of IL-1β on uPAR, suPAR, and PAI-1, particularly in HDPCs. The current understanding suggests the involvement of IL-8 and plasminogen activation molecules in pulpal inflammation. Determining how to control pulp inflammation warrants investigation.

Melatonin is an endocrine generated physiologically mainly by the pineal gland. Melatonin has been shown to exert antioxidant and anti-inflammatory effects as well as to promote wound healing and regeneration of pulpal and periodontal tissues [14,15]. Melatonin can reduce inflammation, regulate cell proliferation, and increase odontoblast activity, but cannot initiate the differentiation of undifferentiated pulp cells [14,15]. Administering melatonin also reduces inflammation and bone resorption in experimentally induced apical lesions in rats [16,17]. These events can be mediated in a receptor-dependent and -independent manner. Interestingly, human dental pulp has been shown to express melatonin and melatonin receptors, melatonin receptor 1 (MT1) and MT2, and their expression is altered during pulpal inflammation [18]. However, little is known about the effect of melatonin on IL-1β, IL-8, and plasminogen activation system molecules in HDPCs.

Regarding the pathogenesis of pulpal inflammation and repair, we hypothesized that during pulpal inflammation, IL-1β may stimulate uPA and uPAR, but inhibit the PAI-1 expression and production of HDPCs. These IL-1β-induced events can be attenuated by melatonin. Clarifying these issues can be helpful for the future prevention and treatment of pulpal/periapical diseases.

2. Materials and methods

2.1. Materials

The cell culture medium and related reagents were obtained from Life Technologies (Thermo Fisher Scientific Ltd., Waltham, MA, USA). Dimethyl sulfoxide (DMSO), LY294002 (a PI3K/Akt inhibitor), U0126 (1,4-diamino-2,3- dicyano-1,4-bis (2-amino phenylthio)butadiene) (a MEK/ERK inhibitor), and SB203580 (a p38 inhibitor), were purchased from Sigma–Aldrich (St. Louis, MO, USA). 5Z-7-Oxozeaenal (a TAK1 inhibitor) was purchased from Tocris Cookson Ltd. (Bristol, UK). Total RNA isolation kits were obtained from Qiagen (Taipei, Taiwan). Polymerase chain reaction (PCR) primers for β-actin (BAC), uPA, uPAR, and PAI-1 were synthesized by Genemed Biotechnologies, Inc. (San Francisco, CA, USA). Enzyme-linked immunosorbent assay (ELISA) kits for uPA and soluble uPAR (suPAR) were obtained from R&D Systems (R&D DuoSet, Minneapolis, MN, USA). Recombinant IL-478
1β and PAI-1 ELISA kits were obtained from PeproTech (PeproTech Asia, Rehovot, Israel).

2.2. Culture of human dental pulp cells

With the approval of the Ethics Committee of National Taiwan University Hospital, caries- and periodontitis-free premolars were extracted from young donors in the Oral and Maxillofacial Clinic after informed consent was obtained. After the teeth were split using a hammer, dental pulp tissues were taken from the pulp chamber. These were then minced into 1-mm³ pieces with a surgical knife and cultured with Dulbecco’s modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS), penicillin, and streptomycin. HDPCs between the 3rd and 8th passages were used in this study [19,20].

2.3. Effects of IL-1β with/without melatonin or other inhibitors on the production of uPA, suPAR, and PAI-1 in dental pulp cells

Briefly, $1 \times 10^5$ cells were inoculated in 24-well culture wells in DMEM containing 10% FBS. After 24 h of attachment, the medium was changed to fresh medium with various concentrations of melatonin (dose study) or IL-1β. In some experiments, melatonin (50, 100, or 200 μg/mL, as indicated) or several signaling inhibitors (such as MEK/ERK inhibitor [U0126], p38 MAPK inhibitor [SB203580], PI3K/Akt inhibitor [LY294002], and TAK1 inhibitor [5Z-7oxozeaenol]) were added before the addition of IL-1β (5 ng/mL), and cells were further incubated for 24 h. The culture medium was collected for enzyme-linked immunosorbent assay (ELISA) analysis of uPA (DuoSet ELISA kit, DY1310, R&D Systems Inc., Minneapolis, MN, USA), suPAR (DuoSet ELISA kit, DY807, R&D Systems), and PAI-1 (PAI-1 ELISA kit, 900-K383, PeproTech Company, Rocky Hill, NJ, USA) based on the manufacturer’s instructions.

2.4. Cell viability assay

Cells were treated as mentioned, and cell viability was estimated using an MTT assay as described previously [21–23]. Briefly, the culture medium was collected for ELISA analysis. Fresh medium (1 mL) containing a final 0.5 mg/mL of MTT was added to each well. Cells were further incubated for 2 h. The culture medium was decanted and then 1 mL of DMSO was added to dissolve the insoluble formazan. The optical density of cells/formazan eluents was measured at a wavelength of OD540 by using a
microplate reader (Dynatech, Golden Valley, MN, USA).

2.5. Effect of IL-1β with/without melatonin on the uPA, uPAR, and PAI-1 mRNA expression of dental pulp cells

Briefly, HDPCs were seeded onto a 6-well culture plate (5 × 10⁵ cells/well) in 2 mL of fresh medium. They were exposed to IL-1β with/without pretreatment with melatonin (50 and 100 μg/mL). After 24 h of exposure, total RNA was isolated using commercially available kits. The RNA was used for reverse transcription (RT) and real-time PCR analysis of the mRNA expression of the pulp cells [22]. Real-time PCR was conducted using the SYBR green method, with 20 μL of reaction mixture containing SYBR master mix (PCR Biosystems Ltd., London, UK), primers, cDNA, and diethyl pyrocarbonate water. Real-Time PCR procedures were performed using the ABI Quantstudio 7 Flex Real-Time PCR System (Life Technologies, Thermo Fisher Scientific Ltd.). The reaction conditions were 95 °C for 2 min for 1 cycle in Stage 1, followed by 95 °C for 5 s and 60 °C for 30 s for 40 cycles in Stage 2. The specific uPA primers were GCCCTCTCTCTCCTCAGAAGAA and GTA-GAGATGTAGT CCTCCTTC; uPAR primers were ATGGAGTGCTCTGTAAGAG and CACAGTGCT GCCAGT CATTAG; and PAI-1 primers were ATGGATTACAGATTGATA and TCAGTAGGACTGACTGTT [24]. The PCR amplification for BAC primers AAGAGAGGCATCCTCACCCT and TACATGGCTGGGGTGTTGAA was used as the control [22]. For quantitative analysis, the delta/ delta cyclic threshold values (ΔΔCt = mean ΔCt [treated] - mean ΔCt [control]) were used to measure changes in the gene expression level. The fold of changes in the experimental groups relative to the control (solvent) group were calculated using the 2⁻ΔΔCt method. The BAC gene expression was used as an internal control in all PCR experiments.

2.6. Immunofluorescent staining of p-ERK, p-p38, p-Akt and p-TAK1 protein expression in HDPCs

Recently we have found the activation of p-ERK, p-p38, p-Akt and p-TAK1 by IL-1β in HDPCs [7,8,20]. To know whether melatonin may inhibit the IL-1β-induced signaling, HDPCs (1 × 10⁵ cells) were seeded onto cover slips in 24-well culture. They were exposed to IL-1β with/without pretreatment and co-incubation by melatonin (50 and 100 μg/mL) for 24 hours. Immunofluorescent staining procedures were performed as before [22].
After removal of culture medium, cells were fixed in 4% paraformaldehyde for 20 min, washed with PBS, and permeabilized in 2% Triton X-100. Cells were then incubated in 0.3% H₂O₂ for 20 min, and blocked by 5% bovine serum albumin to prevent non-specific binding. Thereafter cells were exposed to primary antibodies (p-ERK, p-p38, p-Akt and p-TAK1) at room temperature for overnight. They were then incubated in TRITC-conjugated secondary antibodies (red fluorescence) for 1 hour. Cell nuclei were counterstained by 1:1000 of DAPI (blue fluorescence) for 30 min. Finally, cells in the coverslips were mounted and observed/photographed by a fluorescent microscope (Olympus IX71, Olympus Corporation, Tokyo, Japan).

2.7. Statistical analysis

More than 4 independent experiments were performed. The results are expressed as the mean ± standard error (SE). Statistical analysis of the data was conducted using a paired Student’s t-test. A p-value < 0.05 indicated a statistically significant difference between the groups.

3. Results

3.1. Effect of IL-1β on the uPA, suPAR, and PAI-1 production of HDPCs

This study revealed that IL-1β (>0.1 ng/mL) stimulated the uPA production of HDPCs (Fig. 1A). The stimulatory effect of IL-1β on uPA was

Fig. 3. (A) Effect of melatonin on the viability of HDPCs. Results are expressed as the mean ± SE (% of control = 100). Effect of melatonin on the basal levels of the (B) uPA, (C) suPAR, and (D) PAI-1 production of HDPCs. Results are expressed as the mean ± SE (pg/mL). *denotes a statistically significant difference compared with the control (p < 0.05).
generally similar to that described in our previous report [8]. IL-1β also induced the suPAR production of HDPCs at concentrations higher than 1 ng/mL (Fig. 1B), but IL-1β reduced the PAI-1 production of HDPCs at concentrations higher than 0.1 ng/mL (Fig. 1C).

3.2. Effect of IL-1β on the uPA, uPAR, and PAI-1 mRNA expression of HDPCs

IL-1β has been shown to stimulate the uPA mRNA expression of HDPCs as analyzed using RT-PCR [8]. Accordingly, IL-1β stimulated the uPA mRNA expression of HDPCs as determined by real-time PCR (Fig. 2A). Similarly, IL-1β also provoked the uPAR mRNA expression of HDPCs at concentrations higher than 0.1 ng/mL (Fig. 2B). Conversely, the expression of PAI-1 in HDPCs declined after exposure to IL-1β (Fig. 2C).

3.3. Effect of melatonin on the basal levels of the uPA, suPAR, and PAI-1 production of HDPCs

Melatonin at concentrations below 250 µg/mL showed no marked cytotoxicity to HDPCs (Fig. 3A). Interestingly, melatonin alone appeared to reduce the basal level of uPA production of HDPCs at concentrations higher than 10 µg/mL (Fig. 3B). Melatonin (10–250 µg/mL) alone stimulated the suPAR production of HDPCs with a maximal

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Fig. 4. Effect of melatonin on the IL-1β-induced (A) uPA, (B) suPAR, and (C) PAI-1 production of HDPCs. Results are expressed as the mean ± SE (pg/mL). *denotes a statistically significant difference compared with the control (p < 0.05). #denotes a statistically significant difference compared with the IL-1β-treated group.
stimulation at 100 μg/mL (Fig. 3C). Melatonin alone showed no effect on the PAI-1 production of HDPCs even after 5 days of exposure (Fig. 3D).

3.4. Effect of melatonin on the IL-1β-induced uPA, suPAR, and PAI-1 production of HDPCs

Melatonin showed little effect on IL-1β-induced changes in cell viability (Fig. 4A). However, melatonin (>50 μg/mL) effectively attenuated the IL-1β-induced uPA production of HDPCs (Fig. 4B). Melatonin could not prevent the IL-1β-induced suPAR production of HDPCs, and even enhanced this event (Fig. 4C). Whereas IL-1β reduced PAI-1 production, melatonin showed no marked effect on the PAI-1 production of HDPCs. Melatonin was also unable to prevent the IL-1β-induced decline in PAI-1 production (Fig. 4D).

3.5. Effect of melatonin on the IL-1β-induced uPA and uPAR mRNA expression of HDPCs

We investigated whether melatonin can suppress IL-1β-induced uPA and uPAR expression and found that melatonin markedly prevented IL-1β-induced uPA mRNA expression (Fig. 5A). Unexpectedly, the IL-1β-induced uPAR mRNA expression was mildly enhanced by melatonin (Fig. 5B).

3.6. Effect of inhibitors on the IL-1β-induced uPA, PAI-1, and suPAR production of HDPCs

A previous study found that the uPA and IL-8 production of HDPCs stimulated by IL-1β was mediated via TAK1 and MEK/ERK signaling [8]. Interestingly, we found that the IL-1β-induced uPA production of HDPCs was associated with PI3K/Akt and p38 signaling. These events were attenuated by SB203580 (a p38-MAPK inhibitor) and LY294002 (a PI3K/Akt inhibitor) (Fig. 6A, B).

Few studies have been conducted on the effects of these signaling pathways on the IL-1β-induced uPAR and PAI-1 production of HDPCs. We found that U0126 and 5Z-7oxozeaenol, but not SB203580 attenuated IL-1β-induced suPAR production (Fig. 6C, D, F), and LY294002 enhanced this event (Fig. 6E), suggesting the presence of feedback or compensatory reactions to inflammatory responses.

Regarding the effect on PAI-1, IL-1β mildly reduced the PAI-1 production of HDPCs. Moreover, U0126, LY294002, and 5Z-7oxozeaenol were unable to prevent the IL-1β-induced decline in the PAI-1 production of HDPCs (Fig. 7A, C, D). Interestingly, SB203580 reversed the IL-1β-induced decline in the PAI-1 production of HDPCs (Fig. 7B).

3.7. Effect of melatonin on IL-1β-induced activation of p-ERK, p-p38, p-Akt and p-TAK1 in HDPCs

To know whether the effect of melatonin on IL-1β is associated with its inhibition of signal transduction, we analyzed the activation of p-ERK, p-p38, p-Akt and p-TAK1 by IL-1β and its regulation by melatonin in HDPCs. We found that IL-1β by itself stimulated p-ERK, p-p38, p-Akt and p-TAK1 expression in HDPCs as revealed by increased red fluorescence (Fig. 8A, B, C, D). Melatonin, at concentrations of 50 or 100 μg/mL, attenuated the...
IL-1β-induced activation of p-ERK, p-p38, p-Akt and p-TAK1 (Fig. 8A, B, C, D).

4. Discussion

Bacterial invasion through dental caries, abrasion, and tooth fracture are common causes of pulpal inflammatory diseases. If the dental pulp can adapt to a low level of inflammation, repair and regeneration may occur, but if the pulpal infection is not controlled and the inflammation is severe and continuous, dental pulp necrosis will occur. To eliminate pulpal irritants, control of pulp...
inflammation is crucial to promote healing and successful treatment [25]. As a pro-inflammatory cytokine, IL-1β expression in pulpitis tissues increases in response to caries exposure or bacterial infection [2,3,6]. The effects of IL-1β on plasminogen activation system molecules and prevention via melatonin and other signal transduction inhibitors must be clarified to identify their roles in pulpal/periapical diseases and their effect on fibrinolytic diseases, which are critical factors in inflammation, wound healing, and carcinogenesis. The results may facilitate the prevention and treatment of pulpal/periapical diseases.

Studies have reported that the expression of uPA mRNA and secretion of uPA can be induced by IL-1β in HDPCs [4,8]. But the stimulatory effect of IL-1β on uPA production of HDPCs was similar at concentrations of 0.1–10 ng/mL, indicating IL-1β to be a strong inducer of uPA even at a concentration of 0.1 ng/mL. However, in gingival fibroblasts, exposure to IL-1β stimulates tPA and PAI-2 but exerts no marked effect on uPA and PAI-1 [26], indicating that responses to IL-1β vary among different cell types. Limited information is available on the effects of IL-1β on uPAR/suPAR and PAI-1 in HDPCs. In this study, we found that IL-1β stimulated uPA and uPAR/suPAR but reduced the PAI-1 expression and production of HDPCs. IL-1β and plasminogen are crucial inflammatory mediators for collagen degradation and promote leukocyte infiltration, which are also critical for tissue repair [27]. The autocrine binding of IL-1β-stimulated uPA to the membrane-anchored uPAR/suPAR of fibroblasts or the paracrine binding to uPAR/suPAR exhibited by other cells, such as macrophages, endothelial cells, and neutrophils, may increase the efficacy of activating MMPs and growth factors as well as cleaving plasminogen to generate plasmin, a serine protease. The activation of plasmin further triggers many events including thrombolytic and pericellular extracellular matrix degradation, the breakdown of fibrin-containing clot clot, and angiogenesis, as well as the possibility of cell migration and proliferation and cytokine production in inflammatory processes such...
as rheumatoid arthritis [13]. A decrease in PAI-1 levels further promotes these fibrinolytic events. Elevated uPA levels have been observed in many diseases, such as inflammation, carcinogenesis, angiogenesis, vascular disease (atherosclerosis), and rheumatoid arthritis [13]. These actions are central to tissue inflammation and the repair/regeneration of pulpal and periapical tissues. These results indicate that IL-1β may increase and activate the fibrinolytic systems of human dental pulp during pulpal infection and inflammation.

On the other hand, melatonin is physiologically generated by the pineal gland. It has antioxidant, anti-inflammatory, and immunosuppressive functions and is already used clinically to safely treat insomnia and other neurological diseases [28]. However, little is known about the interactions between melatonin and plasminogen-system-related molecules. Melatonin and its receptors are expressed in human dental pulp [18]. Melatonin reportedly exerts an anti-proliferative effect but induces the differentiation of HDPCs [29]. Conversely, melatonin has been shown to stimulate the proliferation and migration of HDPCs. It also stimulates anti-inflammatory cytokine-TGF-β secretion but cannot induce osteogenic differentiation in HDPCs [15]. In our study, we unexpectedly found that melatonin may effectively suppress the basal level of uPA but stimulates the uPAR expression and suPAR production of HDPCs. Whereas uPA may bind to uPAR and suPAR, the protein interactions of uPAR and suPAR show some differences [13]. Cell surface uPAR may interact with integrins, vitronectin, fMLP-receptors (fMLP-Rs), and growth factor receptors. On the other hand, suPAR binds and activates fMLP-Rs and may regulate the functional activity of MCP-1, RANTES, and SDF1 receptors [13]. The increased expression and production of uPAR and suPAR thus appears to be essential for biological activities including proteolysis; tissue homeostasis; blood coagulation, complement, and plasma kallikrein-kinin cascades; and cell adhesion, migration, and proliferation [13,30]. From the results of the aforementioned studies, melatonin alone may play crucial roles in the control of tissue inflammation, fibrinolysis,
matrix degradation, and cell adhesion, migration, and proliferation during the inflammatory, repair, and regenerative processes of dental pulp.

We have previously reported the preventive effect of melatonin on the areca-nut-induced MMP-9 expression/production of SAS oral cancer cells [31]. Moreover, this study demonstrated that melatonin inhibits the IL-1β-induced uPA expression and production of HDPCs. MEK/ERK, TAK1, and p38 are partly involved in these processes. Melatonin attenuated IL-1β-induced uPA but enhanced uPAR.

Previous studies have found that HDPCs express IL-1 receptor 1 (IL-1R1), but not IL-1R-II [36]. IL-1β differentially activates various signaling molecules such as MEK/ERK, p38, PI3K/Akt, NF-kB, and possibly TAK1 in HDPCs [7,8,36]. The activation of these signaling molecules may affect downstream effective molecules, such as inflammatory mediators such as COX-2/PGE2, IL-8, uPA, and ICAM-1 [7,8,36], and regulate infection, inflammation, and wound healing/repair responses. But little is known about the signal transduction pathways in mediating IL-1β-induced changes of plasminogen activation molecules. Recently MEK/ERK, and TAK1 signaling was found to be associated with IL-1β-induced uPA production [8]. In this study, we found that the IL-1β-induced uPA production of HDPCs can be attenuated by SB203580 and LY294002, implicating its association with p38 and Akt signaling. Similarly IL-1β was shown to stimulate uPA production of gastric cancer cells via ERK and NF-kB signaling [37]. For the regulation of uPAR, protein kinase C (PKC) was found to mediate the IL-1β-induced uPAR in A549 lung cells [38]. Moreover, we found that 5Z-7oxozeaenol and U0126, but not SB203580 and LY294002, could prevent the IL-1β-induced suPAR production of HDPCs. Accordingly, IL-1β-induced uPAR expression in bladder cancer T24 cells is related to ERK and NF-kB signaling [39]. On the other hand, the IL-1β-induced decline of PAI-1 can be prevented by SB203580, but not by U0126, 5Z-7oxozeaenol, or LY294002, implicating the possible differential regulation of signaling pathways. TLR5 is shown to stimulate uPA in HDPCs via NF-kB, MEK/ERK, and p38 signaling [10]. Targeting therapy via the suppression of these signaling molecules may be useful to control tissue inflammation and the fibrinolytic activities of the dental pulp in the future. We therefore tested whether melatonin may prevent the IL-1β-induced signaling molecules and thus partly mediates its antifibrinolytic effect. Interestingly we found the inhibitory effects of melatonin on IL-1β-induced activation of ERK, p38, Akt and TAK1 in this study. Melatonin has been reported to attenuate the LPS-induced proinflammatory mediators (TNF-α, IL-1β, uPA [35]. These results indicate that melatonin may suppress IL-1β-induced uPA/uPAR and uPA/suPAR interactions and inhibit fibrinolysis. Furthermore, increased uPAR and suPAR may promote their interaction with other binding molecules (e.g., integrin, vitronectin, and fMLP-Rs) and thereby control other cell behaviors. Additional studies and models are warranted to further elucidate the antifibrinolytic effects of melatonin and related mechanisms.

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PGE2 etc.) in CRL1999 human vascular smooth muscle cells via inhibition of ERK, p38, and NF-kB signaling [40]. Melatonin also reduced the spinal cord injury in experimental animals by inhibition of ERK, JNK, p38 and TNF-α [41]. Inhibition of these signaling molecules may partly explain the anti-fibrinolytic effect of melatonin.

In conclusion, IL-1β may affect pulpal inflammation, fibrinolytic and granulation tissue formation, and healing via stimulation of uPA and uPAR but reduce PAI-1 in HDPCs. MEK/ERK, TAK1, and p38 are partly involved in these processes (Fig. 9). Melatonin attenuated IL-1β-induced uPA but enhanced uPAR. This suggests that, physiologically, the presence of melatonin in dental pulp may affect the pulpal response to inflammation. Melatonin can potentially be used exogenously in combination with p38 MAPK inhibitor or other inhibitors to serve as a new strategy for a robust anti-inflammation and anti-fibrinolytic therapy in HDPCs in the future. In addition to melatonin, future anti-cytokine or targeting therapy to block the receptor and signal transduction pathways can potentially be used for the treatment of pulpal inflammatory diseases and to facilitate tissue healing and merit for further investigation.

Author contribution

Conceptualization, J.H.J., M.C.C., and B.H.Z.; Methodology, formal analysis, J.H.J., M.C.C., B.H.Z., F.H.C., H.N.L., M.S.L., H.H.C., and Y.H.P.; Investigation, resources, J.H.J., M.C.C., B.H.Z., P.Y.J., H.H.C., and Y.H.P.; Writing – original draft, J.H.J., M.C.C., and B.H.Z.; Writing – review and editing, J.H.J., M.C.C., F.H.C., H.N.L., M.S.L., and H.H.C.; visualization and supervision, J.H.J., M.C.C., and Y.H.P.

Conflict of interest

The authors report no competing interests for this submission.

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