

Melatonin inhibits CXCL10 and MMP-1 production in IL-1 β -stimulated human periodontal ligament cells

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Abstract

Melatonin is a hormone that is mainly secreted by the pineal gland and exhibits a wide spectrum of activities, including antioxidant functions. Melatonin has been detected in gingival crevicular fluid. However, the role of melatonin in periodontal tissue is still uncertain. The aim of this study was to examine the effects of melatonin on inflammatory mediator expression in human periodontal ligament cells (HPDLC).

Interleukin (IL)-1 β induced CXC chemokine ligand (CXCL)10, matrix metalloproteinase (MMP)-1, and tissue inhibitors of metalloproteinase (TIMP)-1 production in HPDLC. Melatonin decreased CXCL10 and MMP-1 production and increased TIMP-1 production in IL-1 β -stimulated HPDLC. Western blot analysis showed that melatonin inhibited p38 mitogen-activated protein kinase (MAPK) and c-jun N-terminal kinase (JNK) phosphorylation, and I κ B- α degradation and phosphorylation in IL-1 β -stimulated HPDLC. These results suggest that melatonin might inhibit Th1 cell migration by reducing CXCL10 production. Moreover, melatonin might inhibit soft tissue destruction by decreasing MMP-1 production in periodontal lesions.

Introduction

Periodontitis is characterized by gingival inflammation, inflammatory cell infiltration, and alveolar bone loss caused by the interaction of host defense mechanisms with a subset of microorganisms that colonize the subgingival environment. Previous reports have revealed that the immune reactions that occur in periodontal lesions are involved in the initiation and progression of periodontal disease [1,2].

CXC chemokine ligand 10 (CXCL10) is a chemokine that plays a role in the immunopathogenesis of inflammatory diseases, such as rheumatoid arthritis [3] and periodontal disease [4]. CXCL10 binds to its receptor, CXC chemokine receptor 3 (CXCR3), and regulates immune responses by activating and recruiting immune cells, including Th1 cells [5]. It is reported that Th1 cells are involved in the exacerbation of periodontal disease [2]. Therefore, we previously examined whether resident periodontal cells produce CXCL10. We reported that certain cytokines induced CXCL10 production in human gingival fibroblasts [6]. However, we did not determine whether there are any factors that can suppress CXCL10 production in periodontal lesions.

Matrix metalloproteinase-1 (MMP-1) is one of the key proteolytic enzymes that degrade types I and III collagen, which are the predominant types of collagen in periodontal tissue. Therefore, MMP-1 might play an important role in destroying periodontal tissue in inflammatory lesions [7]. Tissue inhibitors of metalloproteinase (TIMP) are endogenous inhibitors of MMP. Therefore, the ratio of MMP to TIMP has an important influence on

matrix degradation in inflammatory tissues.

Melatonin, a natural pineal secretory product, is well known for its important functions in circadian rhythm regulation [8], cancer inhibition [9], and seasonal rhythmicity [10]. In addition, melatonin also has broader functions including antioxidant [11] and immunomodulatory roles [12]. However, the effects of melatonin on cytokine expression in periodontal resident cells are unclear.

The aim of this study was to examine the influence of melatonin on CXCL10, MMP-1, and TIMP-1 production in IL-1 β -stimulated human periodontal ligament cells (HPDLC), which are the major resident cells in periodontal tissues. Moreover, we investigated whether melatonin treatment modified the activation of the nuclear factor (NF)- κ B and mitogen-activated protein kinase (MAPK) pathways in IL-1 β -stimulated HPDLC.

Materials and Methods

Cell culture

HPDLC were obtained from Lonza Walkersville Inc. (Walkersville, MD, USA) and grown in Dulbecco's modified Eagle medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS), 1 mmol/L sodium pyruvate (Gibco), and antibiotics (penicillin G: 100 units/ml, streptomycin: 100 μ g/ml) at 37°C in humidified air with 5% CO₂. The cells were used between passage numbers 5 and 10.

CXCL10, MMP-1, and TIMP-1 production in HPDLC

The HPDLC were stimulated with recombinant human IL-1 β (Peprotech, Rocky Hill, NJ,

USA) for 24 hours. The supernatants were collected, and their CXCL10, MMP-1, and TIMP-1 concentrations were examined in triplicate using enzyme-linked immunosorbent assays (ELISA). DuoSet kits (R&D systems, Minneapolis, MN, USA) were used to obtain the CXCL10, MMP-1, and TIMP-1 measurements. All assays were performed according to the manufacturer's recommendations, and CXCL10, MMP-1, and TIMP-1 levels were determined using the standard curve prepared for each assay. In selected experiments, the HPDLC were cultured for 1 hour in the presence or absence of melatonin (Cayman Chemical, Ann Arbor, MI, USA), SB203580 (20 μ M; Santa Cruz Biotechnology, Santa Cruz, CA, USA), SP600125 (20 μ M; Enzo Life Sciences, Plymouth Meeting, PA, USA), or SC514 (20 μ M; Enzo Life Sciences) prior to their incubation with IL-1 β . Melatonin, SB203580, SP600125, and SC514 were dissolved in dimethyl sulfoxide (DMSO) and diluted at least 1 : 2000 in culture media before use. The 2000 times dilution of DMSO had no effect on the viability of the cells.

Western blot analysis

To detect the IL-1 β -induced phosphorylation of signal transduction molecules, Western blot analysis was performed. HPDLC were stimulated with IL-1 β (1 ng/ml) for 15, 30, or 60 minutes after being pretreated (or not) with melatonin (100 μ M or 1000 μ M) for 1 hour. Then, the cells were washed once with ice-cold phosphate-buffered saline, before being incubated on ice for 10 minutes with cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA) supplemented with protease inhibitor cocktail (Sigma). After removing any debris by

centrifugation, the total protein concentrations of the lysates were quantified using the Bradford protein assay. Then, 20- μ g protein samples were fractionated on a 4-20% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel, before being transferred to polyvinylidene difluoride membranes. The membranes were blocked for 1 hour with 1% non-fat dried milk at room temperature and then incubated with phospho-I κ B- α mouse monoclonal antibody (Cell Signaling Technology), phospho-p38 MAPK rabbit monoclonal antibody (Cell Signaling Technology), phospho-extracellular signal-regulated kinase (ERK) rabbit monoclonal antibody (Cell Signaling Technology), phospho-c-jun N-terminal kinase (JNK) rabbit monoclonal antibody (Cell Signaling Technology), p38 MAPK rabbit monoclonal antibody (Cell Signaling Technology), ERK rabbit monoclonal antibody (Cell Signaling Technology), JNK rabbit monoclonal antibody (Cell Signaling Technology), I κ B- α mouse monoclonal antibody (Cell Signaling Technology), or GAPDH rabbit monoclonal antibody (Cell Signaling Technology) overnight at 4°C followed by an appropriate secondary horseradish peroxidase-conjugated antibody (Sigma) for 1 hour at room temperature. Protein bands were visualized using the ECL Prime Western blotting detection system (GE Healthcare, Uppsala, Sweden). Each chemiluminescent signal was quantified using the ImageJ software (version 1.44).

Statistical analysis

Statistical analyses were performed using the Student's *t* test. *P* values of <0.05 were considered significant in Figs. 1 and 4.

Results

Effects of melatonin on CXCL10, MMP-1, and TIMP-1 release from IL-1 β -stimulated HPDLC

It has been reported that IL-1 β induces CXCL10, MMP-1, and TIMP-1 production in HPDLC [13]. Therefore, we first examined if melatonin is able to modulate CXCL10, MMP-1, or TIMP-1 production. As shown in Fig. 1, melatonin treatment decreased both CXCL10 and MMP-1 production in IL-1 β -stimulated HPDLC in a dose-dependent fashion. On the other hand, melatonin (1000 μ M) increased TIMP-1 production in IL-1 β -treated cells.

Effects of melatonin on MAPK phosphorylation in IL-1- β -stimulated HPDLC

Next, we investigated the effects of melatonin on MAPK phosphorylation in IL-1- β -treated HPDLC because it is known that IL-1- β activates MAPK pathways in HPDLC [14,15]. Fig. 2 shows that 1000 μ M melatonin inhibited p38 MAPK and JNK phosphorylation in HPDLC after 60 minutes' IL-1 β -stimulation.

Effects of melatonin on I κ B- α phosphorylation and degradation in IL-1 β -stimulated HPDLC

Next, we examined the effects of melatonin on NF- κ B pathway activation in IL-1 β -stimulated HPDLC because it is known that IL-1 β strongly activates the NF- κ B pathway in HPDLC [16]. Fig. 3 shows that melatonin (100 μ M and 1000 μ M) markedly inhibited I κ B- α phosphorylation and degradation in IL-1 β -stimulated HPDLC.

Effects of p38 MAPK, JNK, and NF- κ B inhibitors on CXCL10, MMP-1, and TIMP-1

production in IL-1 β -stimulated HPDLC

Figs. 2 and 3 show that melatonin inhibited p38 MAPK, JNK, and NF- κ B activation in HPDLC that had been stimulated with IL-1 β . Next, we examined whether p38 MAPK, JNK, or NF- κ B are involved in CXCL10, MMP-1, or TIMP-1 release from IL-1 β -stimulated HPDLC using inhibitors. Fig. 4 shows that the p38 MAPK, JNK, and NF- κ B inhibitors significantly inhibited CXCL10 production in IL-1 β -treated HPDLC. On the other hand, the p38 MAPK inhibitor did not modulate MMP-1 release from HPDLC although the JNK and NF- κ B inhibitors markedly suppressed MMP-1 production in IL-1 β -stimulated HPDLC. None of the inhibitors had any effect on TIMP-1 production in IL-1 β -treated cells.

Discussion

In this study, we demonstrated, for the first time, the anti-inflammatory effects of melatonin on HPDLC.

Previous studies examined the expression of melatonin in periodontal tissue. Srinath et al. reported that melatonin was expressed in gingival crevicular fluid, and the level of melatonin was lower in subjects with periodontal disease than in healthy subjects [17]. Similarly, Almughrabi et al. found that the melatonin levels in gingival crevicular fluid and saliva were lower in patients with chronic periodontitis or aggressive periodontitis than in those with gingivitis and healthy subjects [18]. They suggested that melatonin might have a protective role against periodontal disease. We consider that the anti-inflammatory effects of melatonin might be important for the initiation and progression of periodontal disease.

Various researchers have described the anti-inflammatory effects of melatonin on periodontal resident cells. Choi et al. reported that melatonin inhibited nitric oxide and IL-6 production in *Prevotella intermedia*, which is a major cause of inflammatory reactions in periodontal tissue,-stimulated murine macrophages [19]. Gómez-Florit et al. demonstrated that melatonin treatment inhibited MMP-1 and IL-6 production in non-stimulated human gingival fibroblasts [20]. The above results and our findings show that melatonin modulates the anti-inflammatory effects of various kinds of cells in periodontal lesions and suppresses the progression of periodontal inflammation.

There have only been a few *in vitro* studies about the effects of melatonin on CXCL10 production. Ban et al. reported that microarray analysis showed that melatonin inhibited CXCL10 mRNA expression in lipopolysaccharide(LPS)-activated RAW 264.7 macrophages, which agrees with our findings [21]. Melatonin might inhibit Th1 cell migration and accumulation, and hence, inhibit CXCL10 production by resident periodontal cells and immune cells in diseased periodontal tissue.

There have been several reports about the effects of melatonin on MMP and TIMP expression. Qin et al. reported that melatonin inhibited MMP-9 production by IL-1 β -stimulated human umbilical vein endothelial cells [22], and Esposito et al. found that the activity and expression of MMP-9 and MMP-2 were reduced by melatonin during experimental colitis [23]. Recently, Gómez-Florit et al. demonstrated that melatonin treatment inhibited MMP-1 production and enhanced TIMP-1 expression in non-stimulated human gingival fibroblasts [20]. They did not

examine the effects of melatonin on cytokine or bacteria-stimulated gingival fibroblasts. However, taking these results together with our findings it is suggested that melatonin prevents tissue degradation by inhibiting MMP-1 production and increasing TIMP-1 production in gingival fibroblasts and periodontal ligament cells in diseased periodontal tissue.

Some previous studies examined the effects of melatonin on MAPK activation. Shin et al. reported that melatonin markedly decreased the phosphorylation of MAPK, including p38 MAPK, ERK, and JNK, induced by epidermal growth factor stimulation in human airway epithelial cells [24]. On the other hand, Xia et al. showed that it had no effect on Toll-like receptor 4-mediated phosphorylation of p38 MAPK, ERK, and JNK in LPS-stimulated RAW 264.7 macrophages [25]. In the current study, we demonstrated that melatonin inhibited p38 MAPK and JNK activation, whereas ERK phosphorylation was not affected by melatonin treatment in IL-1 β -stimulated HPDLC. We consider that the effects of melatonin on MAPK activation are dependent on the type of cells and stimulants involved.

Previous reports have described the effects of melatonin on NF- κ B activation. Xiao et al. reported that melatonin inhibited I κ B- α degradation in LPS-stimulated RAW264.7 macrophages [25]. Qin et al. found that melatonin inhibited the nuclear translocation of NF- κ B p65 in IL-1 β -stimulated human umbilical vein endothelial cells [22]. Taking these results together with ours, it is suggested that melatonin decreases inflammatory mediator expression by suppressing NF- κ B activation in certain types of cells, including macrophages,

endothelial cells, and mesenchymal cells.

The present study demonstrated that melatonin suppressed IL-1 β -induced CXCL10 and MMP-1 production, and enhanced TIMP-1 production in HPDLC. In addition, we revealed that melatonin inhibited p38 MAPK and JNK phosphorylation and I κ B- α degradation in IL-1 β -stimulated HPDLC. These results indicate that melatonin inhibits the initiation and progression of periodontal disease by controlling chemokine, MMP, and TIMP production in periodontal lesions.

Acknowledgements

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Conflicts of interest

The authors report that there are no conflicts of interest related to this study.

Figure legends

Fig. 1. Effects of melatonin on CXCL10, MMP-1, and TIMP-1 production in IL-1 β -stimulated HPDLC

HPDLC were treated with melatonin (1, 10, 100, or 1000 μ M) for 1 hour, before being stimulated with IL-1 β (1 ng/ml). The supernatants were collected after 24 hours' incubation. The concentrations of CXCL10, MMP-1, and TIMP-1 in the supernatants were assessed using ELISA. The results are shown as the mean and SD of one representative experiment

performed in triplicate. The error bars represent SD values. * = $P < 0.05$, significantly different from the IL-1 β -stimulated HPDLC that were not pretreated with melatonin

Fig. 2. Effects of melatonin on IL-1 β -induced p38 MAPK, ERK, and JNK phosphorylation in HPDLC

The cultured cells were treated with melatonin (100 or 1000 μ M) for 60 min and then stimulated with 1 ng/ml IL-1 β for 15, 30, or 60 min. The cells were lysed in lysis buffer containing protease inhibitors, and the phosphorylation of p38 MAPK, ERK, and JNK was assessed using Western blot analysis. A representative Western blot that shows the phospho-p38 MAPK, total p38 MAPK, phospho-ERK, total ERK, phospho-JNK, and total JNK levels detected in the HPDLC during three independent experiments is shown. The bar graphs of phospho-p38 MAPK, phospho-ERK, and phospho-JNK expression were normalized to total p38 MAPK, total ERK, and total JNK, respectively.

Fig. 3. Effects of melatonin on IL-1 β -induced I κ B- α phosphorylation and degradation

The cultured cells were pretreated with melatonin (100 or 1000 μ M) for 60 min and then stimulated with 1 ng/ml IL-1 β for 15, 30, or 60 min. The cells were lysed in lysis buffer containing protease inhibitors, and the phosphorylation and degradation of I κ B- α were assessed using Western blot analysis. A representative Western blot that indicates the phospho-I κ B- α , total I κ B- α , and GAPDH levels detected in the HPDLC during three independent experiments is shown. The bar graphs of phospho-I κ B- α or total I κ B- α expression were normalized to GAPDH.

Fig. 4. Effects of signal transduction inhibitors on IL-1 β -stimulated CXCL10, MMP-1, and TIMP-1 release by HPDLC

The cells were pre-incubated with SB203580 (20 μ M), SP600125 (20 μ M), or SC514 (20 μ M) for 1 hour and then incubated with IL-1 β (1 ng/ml). After 24 hours' incubation, the supernatants were collected, and their CXCL10, MMP-1, and TIMP-1 levels were measured using ELISA. The results are shown as the mean and SD of one representative experiment performed in triplicate. The error bars show the SD of the values. * = $P < 0.01$ significantly different from the IL-1 β -stimulated HPDLC that were not treated with inhibitors

References

1. Teng YT. 2003. The role of acquired immunity and periodontal disease progression. *Crit Rev Oral Biol Med.* 14: 237-52
2. Taubman MA, Valverde P, Han X, Kawai T. 2005. Immune response: the key to bone resorption in periodontal disease. *J Periodontol.* 76: 2033-41.
3. Lee EY, Lee ZH, Song YW. 2013. The interaction between CXCL10 and cytokines in chronic inflammatory arthritis. *Autoimmun Rev.* 12:554-7.
4. Garlet GP, Martins W Jr, Ferreira BR, Milanezi CM, Silva JS. 2003. Patterns of chemokines and chemokine receptors expression in different forms of human periodontal disease. *J Periodontal Res.* 38: 210-7.
5. Bonecchi R, Bianchi G, Bordignon PP, D'Ambrosio D, Lang R, Borsatti A, Sozzani S, Allavena P, Gray PA, Mantovani A, Sinigaglia F. 1998. Differential

- expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J Exp Med.* 187:129-34.
6. Hosokawa Y, Hosokawa I, Ozaki K, Nakae H, Matsuo T. 2009. Cytokines differentially regulate CXCL10 production by interferon-gamma-stimulated or tumor necrosis factor-alpha-stimulated human gingival fibroblasts. *J Periodontal Res.* 44:225-31.
 7. Aiba T, Akeno N, Kawane T, Okamoto H, Horiuchi N. 1996. Matrix metalloproteinases-1 and -8 and TIMP-1 mRNA levels in normal and diseased human gingivae. *Eur J Oral Sci.* 104:562-9.
 8. Zawilska J, Nowak JZ. 1991. Regulation of melatonin biosynthesis in vertebrate retina: involvement of dopamine in the suppressive effects of light. *Folia Histochem Cytobiol.* 29: 3-13.
 9. Reiter RJ. 2004. Mechanisms of cancer inhibition by melatonin. *J Pineal Res.* 37: 213-4.
 10. Arendt J. 1986. Role of the pineal gland and melatonin in seasonal reproductive function in mammals. *Oxf Rev Reprod Biol.* 8: 266-320.
 11. Yang Y, Sun Y, Yi W, Li Y, Fan C, Xin Z, Jiang S, Di S, Qu Y, Reiter RJ, Yi D. 2014. review of melatonin as a suitable antioxidant against myocardial ischemia-reperfusion injury and clinical heart diseases. *J Pineal Res.* 57: 357-66.

12. Calvo JR, Rafii-el-Idrissi M, Pozo D, Guerrero JM. 1995. Immunomodulatory role of melatonin: specific binding sites in human and rodent lymphoid cells. *J Pineal Res.* 18: 119-26.
13. Hosokawa Y, Hosokawa I, Shindo S, Ozaki K, Matsuo T. 2015. Calcitriol Suppressed Inflammatory Reactions in IL-1 β -Stimulated Human Periodontal Ligament Cells. *Inflammation.* 38: 2252-8.
14. Oikawa A, Kobayashi M, Okamatsu Y, Shinki T, Kamijo R, Yamamoto M, Hasegawa K. 2007. Mitogen-activated protein kinases mediate interleukin-1 β -induced receptor activator of nuclear factor- κ B ligand expression in human periodontal ligament cells. *J Periodontal Res.* 42: 367-76.
15. Murayama R, Kobayashi M, Takeshita A, Yasui T, Yamamoto M. 2011. MAPKs, activator protein-1 and nuclear factor- κ B mediate production of interleukin-1 β -stimulated cytokines, prostaglandin E₂ and MMP-1 in human periodontal ligament cells. *J Periodontal Res.* 46: 568-75.
16. Zhu L, Wu Y, Wei H, Yang S, Zhan N, Xing X, Peng B. 2012. Up-regulation of IL-23 p19 expression in human periodontal ligament fibroblasts by IL-1 β via concurrent activation of the NF- κ B and MAPKs/AP-1 pathways. *Cytokine.* 60: 171-8.
17. Srinath R, Acharya AB, Thakur SL. 2010. Salivary and gingival crevicular fluid melatonin in periodontal health and disease. *J Periodontol.* 81: 277-83.

18. Almughrabi OM, Marzouk KM, Hasanato RM, Shafik SS. 2013. Melatonin levels in periodontal health and disease. *J Periodontal Res.* 48: 315-21.
19. Choi EY, Jin JY, Lee JY, Choi JI, Choi IS, Kim SJ. 2011. Melatonin inhibits *Prevotella intermedia* lipopolysaccharide-induced production of nitric oxide and interleukin-6 in murine macrophages by suppressing NF- κ B and STAT1 activity. *J Pineal Res.* 50:197-206.
20. Gómez-Florit M, Ramis JM, Monjo M. 2013. Anti-fibrotic and anti-inflammatory properties of melatonin on human gingival fibroblasts in vitro. *Biochem Pharmacol.* 86: 1784-90.
21. Ban JY, Kim BS, Kim SC, Kim DH, Chung JH. 2011. Microarray Analysis of Gene Expression Profiles in Response to Treatment with Melatonin in Lipopolysaccharide Activated RAW 264.7 Cells. *Korean J Physiol Pharmacol.* 15: 23-9.
22. Qin W, Lu W, Li H, Yuan X, Li B, Zhang Q, Xiu R. 2012. Melatonin inhibits IL1 β -induced MMP9 expression and activity in human umbilical vein endothelial cells by suppressing NF- κ B activation. *J Endocrinol.* 214: 145-53.
23. Esposito E, Mazzon E, Riccardi L, Caminiti R, Meli R, Cuzzocrea S. 2008. Matrix metalloproteinase-9 and metalloproteinase-2 activity and expression is reduced by melatonin during experimental colitis. *J Pineal Res.* 45: 166-73.
24. Shin IS, Park JW, Shin NR, Jeon CM, Kwon OK, Lee MY, Kim HS, Kim JC,

- Oh SR, Ahn KS. 2014. Melatonin inhibits MUC5AC production via suppression of MAPK signaling in human airway epithelial cells. *J Pineal Res.* 56: 398-407.
25. Xia MZ, Liang YL, Wang H, Chen X, Huang YY, Zhang ZH, Chen YH, Zhang C, Zhao M, Xu DX, Song LH. 2012. Melatonin modulates TLR4-mediated inflammatory genes through MyD88- and TRIF-dependent signaling pathways in lipopolysaccharide-stimulated RAW264.7 cells. *J Pineal Res.* 53: 325-34.

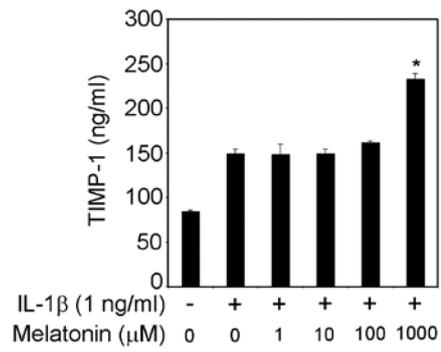
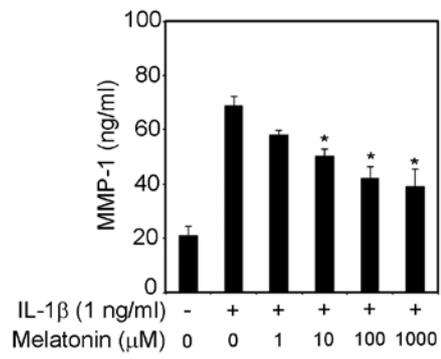
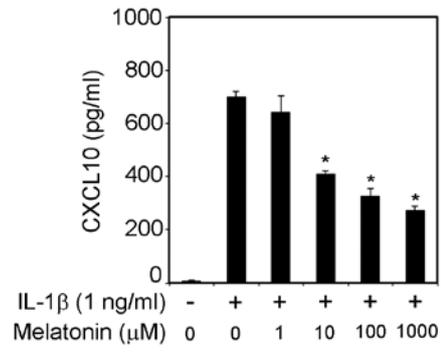


Fig. 1

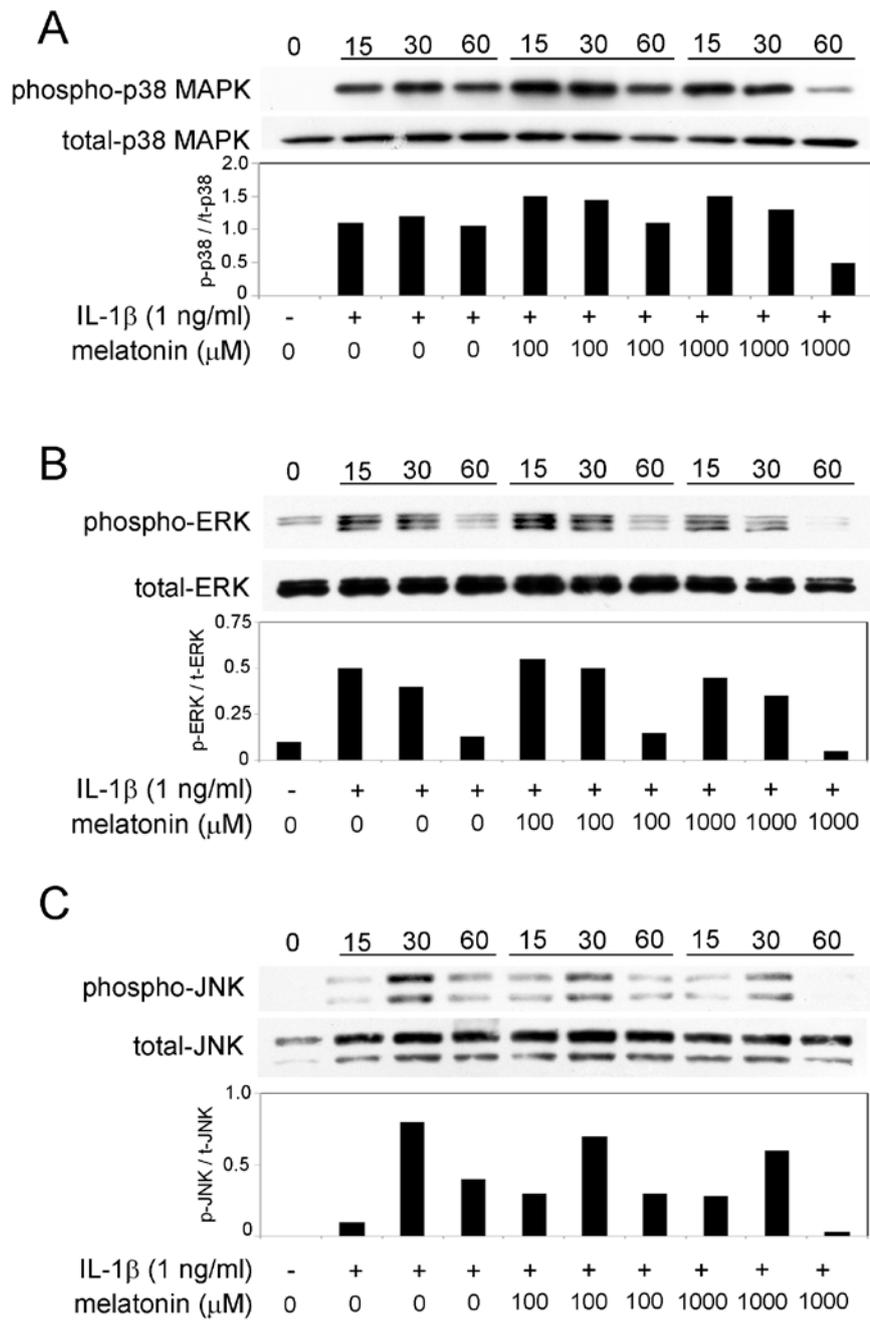


Fig. 2

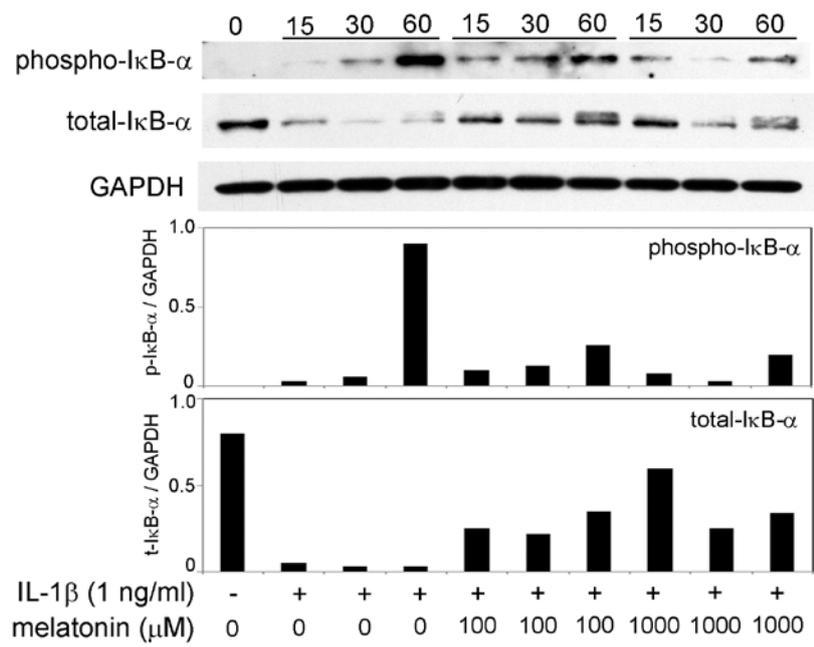


Fig. 3

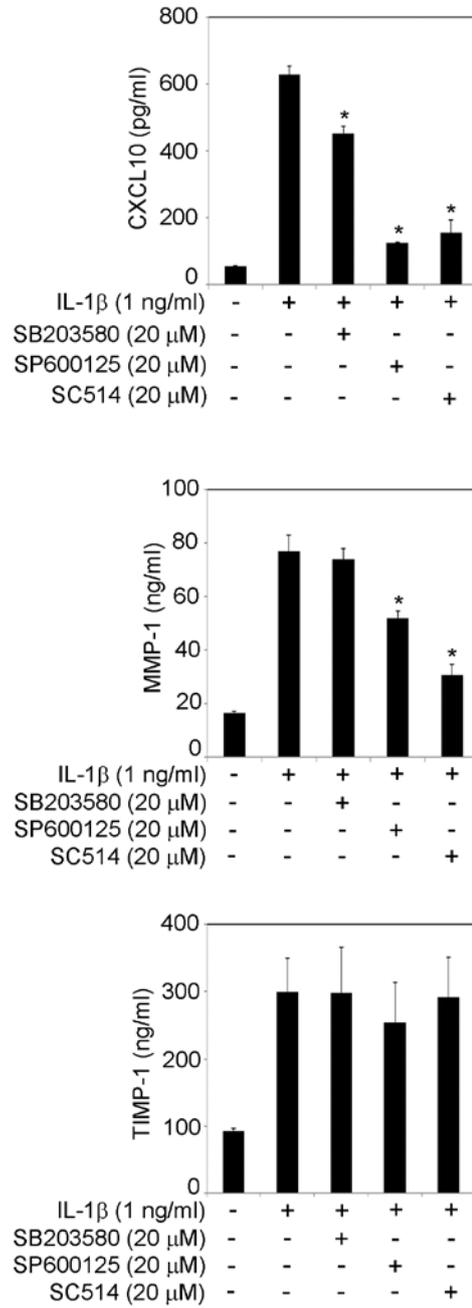


Fig. 4