Melatonin inhibits IL1β-induced MMP9 expression and activity in human umbilical vein endothelial cells by suppressing NF-κB activation

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Abstract

Matrix metalloproteinases (MMPs) have been involved in inflammatory and degradative processes in pathologic conditions. The purpose of this study was to investigate the protective effect of melatonin in human umbilical vein endothelial cell (HUVEC) monolayer permeability and the regulation of MMP9 induced by interleukin 1β (IL1β (IL1B)) in HUVECs. Protection studies were carried out with melatonin, a well-known antioxidant and antiinflammatory molecule. MMP9 expression was increased with IL1β induction in HUVECs. Melatonin showed a barrier-protective role by downregulation of MMP9 and upregulation of tissue inhibitor of metalloproteinase-1 expression in HUVECs. Meanwhile, melatonin also decreased sodium fluorescein permeability and counteracted the downregulation of vascular endothelial cadherin and occludin expression in HUVECs. During inflammatory stimulus, nuclear factor-κB (NF-κB) plays a significant role in regulating MMP genes expression, thus the function of NF-κB in HUVECs’ barrier disruption was investigated. IL1β induced nuclear translocation of NF-κB in HUVECs and regulated MMP9 expression. However, NF-κB translocation into the nucleus was inhibited significantly by melatonin. Our results show that melatonin decreases the permeability of monolayer endothelial cell induced by IL1β. At the same time, melatonin decreased the expression and activity of MMP9 by a NF-κB-dependent pathway in HUVECs induced by IL1β.


Introduction

Matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases, are known to play vital roles in the degradation of basement membranes and the extracellular matrix (Birkedal-Hansen et al. 1993, Visse & Nagase 2003). MMPs are involved in the development processes of experimental colitis (Esposito et al. 2008b), endometriosis (Paul et al. 2008), liver injury (Mishra et al. 2011), traumatic experimental spinal cord injury (Esposito et al. 2008a), acute ischemic stroke (Ramos-Fernandez et al. 2010), and neurodegenerative diseases (Reyes et al. 2009, Rosenberg 2009, Bauer et al. 2010). Meanwhile, many studies also show that MMPs have contributed to tumor invasion, metastasis, and angiogenesis (Toth & Fridman 2001, Zhang et al. 2003, Kessenbrock et al. 2010, Pratheeshkumar & Kuttan 2011).

MMP2 and MMP9, also called collagenases, are well-known to degrade type IV collagen, gelatin, and elastin (Murphy et al. 1994, Shipley et al. 1996). The proteolytic activity of MMPs can be regulated at several levels: gene expression, secretion, and conversion from zymogen to active enzyme (Kessenbrock et al. 2010, Mishra et al. 2011). All the activated MMPs (including MMP2 and MMP9) are specifically inhibited by endogenous inhibitors, tissue inhibitor of metalloproteinases (TIMPs; Visse & Nagase 2003, Kessenbrock et al. 2010). MMP2 and MMP9 can be induced in multiple cells by inflammatory cytokines including interleukin 1β (IL1β (IL1B)) and tumor necrosis factor α (TNFα; Mackay et al. 1992, Li et al. 1999, Siwik et al. 2000, Jones et al. 2005, Redondo-Munoz et al. 2006, Mountain et al. 2007). Our pervious study showed that IL1β increased the permeability of the human umbilical vein endothelial cell (HUVEC) monolayer by damaging adherens junctions and enlarging the junctions cleft width (Yuan et al. 2011). We presume that the disruption of HUVECs monolayer permeability induced by IL1β may be connected with MMPs. It was reported that MMP9 secreted in different cells degraded tight junctions and adherens junctions, which disrupted the integrity of the endothelial barrier resulting in an increase in the vascular permeability (Wachtel et al. 1999, Alexander & Elrod 2002).
Nuclear factor-κB (NF-κB) is a dimer of members of the Rel family that consists of a group of five proteins (Takada & Aggarwal 2003). A heterodimer of p65 and p50 subunits, which was the first NF-κB molecule described, is inhibited by IkB protein in unstimulated cells (DiDonato et al. 1997). NF-κB activated by TNFα has been observed in human dermal fibroblasts, which can activate pro-MMP2 (Han et al. 2001).

Melatonin, the hormone produced by the pineal gland and having binding sites in the brain, is released into the cerebrospinal fluid and circulation and exerts various important biological effects (Williams 1989, Tan et al. 2010). Melatonin demonstrates neuroprotective effects in models of cerebrovascular diseases through either antioxidant or antiinflammatory effects or inhibition of MMPs (Tai et al. 2010, Swarnakar et al. 2011). Melatonin shows the inhibition of forskolin-stimulated adenylate cyclase in ovine pars tuberalis cells (Morgan et al. 1991). Previously, our results showed that the barrier-protective effects of melatonin on endothelial cells were mediated by Rac activation and lead to the enhancement of adherens junctions (Yuan et al. 2011). Whether melatonin protects the integrity of HUVECs monolayer involved MMPs remains unknown. Herein we studied MMP expressions in HUVECs in response to IL1β and then investigated the protective effect of melatonin on HUVECs’ barrier disrupted by MMPs.

Material and Methods

Preparation of cells and culture of HUVECs

Immediately after term-delivery, primary HUVECs were isolated and cultured from fresh human umbilical cord veins (derived from Beijing Hospital, Beijing, China) by collagenase digestion as previously described (Jaaf et al. 1973, Yuan et al. 2011). The cells were grown in fibronectin-coated tissue culture flasks (Corning, Unioning City, CA, USA) in endothelial cell medium (ECM; Sciencell, San Diego, CA, USA) and maintained in humidified air containing 5% (v/v) CO2 at 37 °C in incubator. To reduce the risk of the HUVECs’ functional properties in vitro environment, they were used for no more than five passages.

<table>
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<th>mRNA</th>
<th>Sense primer</th>
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<td>299</td>
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</table>

MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Evaluation of the monolayer permeability of HUVECs

The flux of sodium fluorescein (Na-F) across the endothelial monolayers in a Transwell system (0.4 μm pore size polyester membrane inserts; Corning) was determined as previously described (Nakagawa et al. 2009). When HUVECs got confluent, HUVECs were treated with IL1β (10 ng/ml) with or without melatonin (10 μmol/l) in serum-free medium for 24 h. Briefly, 10 μg/ml Na-F (molecular weight (MW): 376 Da) was added to the upper inserts of the Transwell system. At 0, 5, 15, 30, 60, 90, and 120 min, the concentration of Na-F was measured in the lower chamber.
Melatonin inhibits MMP9 in HUVECs  ·  W QIN, W LU and others

10 µl of medium were aspirated from the lower compartments and replaced with the same volume of fresh serum-free medium. The value of fluorescence that passed through the inserts was determined by fluorescence multiwall plate reader Synergy4 (BioTek, Winooski, VT, USA; excitation wavelength: 485 nm, emission wavelength: 535 nm).

Immunostaining

To characterize the cultures after washing and fixation, endothelial cells were incubated with anti-von Willebrand factor (vWF) rabbit polyclonal antibody (Santa Cruz Biotechnology) and FITC-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology). The primary antibody was used in a dilution 1:100. To counterstain cell nuclei 4,6-diamidino-2-phenylindole (DAPI) was used according to the manufacturer’s instructions.

To stain endothelial junctional proteins, HUVEC monolayers cultured on fibronectin-coated 35-mm cell culture dishes were stained for occludin, claudin-5, zonula occludens-1 (ZO-1), and vascular endothelial cadherin (VE-cadherin). The cultures were washed in PBS and fixed with ethanol for 30 min at 4°C (occludin and claudin-5), ethanol (95 vol%)-acetic acid (5 vol%) for 10 min at −20°C (ZO-1) or with 4% paraformaldehyde in PBS for 15 min and then permeabilized for 10 min with 0.1% Triton X-100 at room temperature (VE-cadherin and anti-NF-κB/p65). Cells were blocked with 3% BSA and incubated with primary antibodies anti-occludin, anti-claudin-5, anti-ZO-1, anti-VE-cadherin, and anti-NF-κB/p65 (Santa Cruz Biotechnology) overnight. Incubation with secondary antibody FITC-conjugated or TRITC-conjugated anti-rabbit and anti-mouse immunoglobulins (both from Santa Cruz Biotechnology) lasted for 1 h at room temperature. Incubation with secondary antibody FITC-conjugated or TRITC-conjugated anti-rabbit and anti-mouse immunoglobulins (both from Santa Cruz Biotechnology) lasted for 1 h at room temperature. Images were acquired by fluorescence microscope (Olympus Corporation, Monolith, Japan) with appropriate filter sets.

Reverse transcriptase-PCR

Total RNA from HUVECs was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions and quantified by measuring the absorbance at 260 nm using Nanodrop2000 (Thermo Fisher Scientific, MA, USA). cDNA was synthesized using 1 µg of total RNA in a 25 µl reaction volume using TIANscript M-MLV reverse transcriptase (Tiangen Biotech, Beijing, China) with an oligo (dT)15 primer. The cDNA (2 µl) was then amplified in 25 µl PCR reaction volume for 30 cycles of denaturation (94°C for 30 s), annealing (55°C for MMP9, 55°C for TIMP1, 54°C for TIMP2, and 52°C for GAPDH respectively for 30 s each), and extension (72°C for 60 s). The primers used to measure these mRNA levels are listed in Table 1. A 5 µl volume of each PCR product was observed by electrophoresis in 2% agarose gels, visualized by ethidium bromide staining and the band intensities were quantified using ImageJ program (http://rsb.info.nih.gov/ij).

Gelatin zymography

For assay of MMP2 and MMP9 enzymatic activity, the conditioned medium of HUVECs was analyzed by gelatin zymography (Toth & Fridman 2001). Serum-free conditioned media were subjected to 10% SDS–PAGE containing 1 ng/ml gelatin (Sigma–Aldrich) under non-reducing conditions. Equal protein content of samples was mixed with 1% (w/v) SDS sample buffer under nonreducing conditions and loaded onto gels. Gels were run at 125 V until the Bromophenol Blue dye had run off the gels. Gels were washed twice with 2.5% (v/v) Triton X-100 and subsequently incubated in developing buffer containing 50 mmol/l Tris–HCl (pH 7.8), 0.2 M NaCl, 5 mmol/l CaCl2 and 0.02% sodium azide for 42 h at 37°C. Gels were stained with 0.1% Coomassie Blue R-250 followed by destaining in different constituents of methanol/acetic acid (30% (v/v)/10% (v/v), 30 min; 20% (v/v)/10% (v/v), 1 h; and 10% (v/v)/5% (v/v), 2 h).

![Figure 1](image-url)
Gelatinolytic activity appeared as transparent bands on a blue background and was determined by scanning the bands using BINDA 2020D. The band intensities were quantified using ImageJ program (http://rsb.info.nih.gov/ij).

Western blot analysis

To determine the levels of protein expression in the membrane, cytoplasm or the nucleus, we prepared extracts using Membrane Protein Extraction Kit (Thermo Fisher Scientific) and NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific). Equal amounts of protein were separated on 10% SDS–PAGE electrophoresis and transferred onto nitrocellulose (NC) membranes (Pall Life Sciences, Port Washington, NY, USA). The NC membranes were blocked with 5% nonfat milk in TBST (10 mmol/l Tris–HCl (pH 7.5), 150 mmol/l NaCl, 0.05% Tween-20) for 1 h at room temperature and then incubated with the appropriate primary antibody at dilutions in blocking buffer at 4 °C overnight. The membranes were washed the next day, and primary antibodies were detected with the appropriate HRP–conjugated secondary antibodies at dilutions for 1 h at room temperature. The membranes were washed three times and then developed using an ECL Kit, followed by exposing to X-ray films. The band intensities were quantified using ImageJ program (http://rsb.info.nih.gov/ij).

Statistical analysis

All data presented are mean±s.d. Statistical significance was analyzed using the statistical program SPSS 17.0 for windows (SPSS, Inc., Chicago, IL, USA). Differences with a P value <0.05 were considered to be statistically significant. All experiments were repeated at least three times.

Results

HUVECs obtained by immunomagnetic separation grew in nonoverlapping continuous monolayers and showed large, homogenous, closely opposed and polygonal cells with an oval, centrally located nucleus and indistinct cell borders. By phase-contrast microscopy, the HUVECs formed typical ‘cobblestone’ morphology and appeared to connect separate cells when the monolayers were confluent and were positive immunostaining for vWF, a marker for endothelium (data not shown).

To investigate the effect of melatonin on expressions of MMP9, MMP2, TIMP1, and TIMP2 in HUVECs, we performed RT-PCR analyses (Fig. 1A). IL1β-treated group exhibited higher MMP9 expression relative to the control group, whereas melatonin pretreatment significantly reduced MMP9 mRNA level relative to IL1β-treated group (Fig. 1A and B). However, we observed no difference in MMP2 mRNA levels in the IL1β-treated or IL1β + Mel groups relative to the control group (Fig. 1A and C). Furthermore, TIMP1 mRNA was downregulated in the IL1β-treated group compared with the control group (Fig. 1A and D). Compared with the IL1β-treated group, melatonin pretreatment significantly increased TIMP1 mRNA levels (Fig. 1A and D).
No difference in TIMP2 mRNA levels was observed between the control groups and IL1β-treated or IL1βCMel groups (Fig. 1A and E).

To monitor the MMP2 and MMP9 enzymatic activities and protein levels, we performed gelatin zymography and western blot analysis (Fig. 2A and D). MMP9 enzymatic activity was significantly increased in IL1β-treated group compared with the control group, but it was effectively suppressed by melatonin pretreatment in the IL1βCMel group (Fig. 2A and B). However, when compared with the control group, there was no significant distinction in MMP2 enzymatic activity between IL1β-treated and IL1βCMel groups (Fig. 2A and C). Meanwhile, western blot analysis demonstrated that IL1β significantly increased MMP9 protein level but had no significant effect on MMP2 protein level (Fig. 2D, E, and F). Pretreatment with melatonin inhibited MMP9 protein expression but had no significant effect on MMP2 protein level (Fig. 2D, E, and F).

Secreted MMP activity is highly modulated by its endogenous inhibitor, TIMP. We examined the expression of TIMP levels (TIMP1 and TIMP2) in HUVECs using western blot analysis. When compared with the control group, TIMP1 levels were significantly reduced in the IL1β-treated group. However, melatonin pretreatment significantly increased the expression of TIMP1 in the IL1βCMel group (Fig. 2D and G). Nevertheless, there were no changes detected in TIMP2 protein levels between IL1β-treated and IL1βCMel groups (Fig. 2D and H).

Endothelial monolayer permeability was determined with Na-F by the Transwell assay. The permeability of the endothelial monolayers in the IL1β-treated group was obviously increased at different time points compared with the control group (Fig. 3). However, the permeability to Na-F in the group pretreated with melatonin for 30 min was significantly reduced (Fig. 3).

The influence of MMPs on adherens and tight junctional proteins has been involved in the integrity of endothelial barrier. By immunofluorescence staining, the IL1β-treated group, in which MMP activities were higher, displayed an obvious disruption of adherens junctions (VE-cadherin) and tight junctions (occludin) as shown in Fig. 4A and B. However, melatonin pretreatment attenuated the disruption of VE-cadherin and occludin (Fig. 4A and B). Meanwhile, we also found that the other tight junctional proteins claudin-5 and ZO-1 were disrupted in the IL1β-treated group, while they were protected by melatonin pretreatment in the IL1βCMel group (Fig. 4C and D). Next, we examined whether increased MMP9 changed VE-cadherin and occludin protein levels. The protein levels of VE-cadherin and occludin in the IL1β-treated group were decreased by 34.7% and 67.2% respectively, while the reductions were largely reversed by melatonin in the IL1βCMel group (Fig. 5A, B, and C).

The above results showed that the reduced expressions of
VE-cadherin and occludin were associated with MMP9 enzymatic activity, while pretreatment with melatonin could counteract with the effect of MMP9.

NF-κB/p65, which is one of the NF-κB protein family, transferred to the nucleus and bound to a specific sequence in the genome, resulting in the regulation of MMP transcription when cells received inflammatory stimuli. Our western blot results showed that the activated NF-κB/p65 was increased in the IL1β-treated group, while pretreatment with melatonin could obviously inhibit the nuclear translocation of NF-κB/p65 (Fig. 6A and B). We also analyzed the cellular location changes of NF-κB/p65 after IL1β treated with or without melatonin pretreated by the immunostaining method. We observed that NF-κB/p65 was located in the cytoplasm in the control group, while in IL1β-treated cells, NF-κB/p65 translocated into the nucleus. Pretreatment with melatonin clearly suppressed the p65 location changes visible on the immunofluorescence (Fig. 6C).

**Discussion**

The observations of this study can be summarized as follows. First, MMP9 mRNA and protein levels are upregulated in HUVECs under IL1β stimulation, while its natural inhibitor TIMP1 mRNA and protein levels are downregulated, whereas there are no changes in MMP2 mRNA and protein levels. Second melatonin pretreatment reduces the expression levels of MMP9 mRNA and protein, but increases TIMP1 mRNA and protein levels. Third increased MMP9 activity disturbs the HUVECs adherens junctions and tight junctions and increases the permeability of HUVECs to Na-F. Fourth melatonin attenuates the deleterious effect of MMP9 on cell–cell junctions of HUVECs and protects the permeability of HUVECs. Finally, melatonin prevents the nuclear translocation of NF-κB/p65 and further reduces MMP9 gene expression. Considering that MMP9 fractured the adherens junctions and tight junctions of HUVECs and increased permeability to Na-F these results suggest that MMPs induced by inflammatory cytokines IL1β might be a potential mechanism that affects endothelial barrier function. This research for the first time demonstrates that MMP9 plays important roles in endothelial integrity disruption.

MMPs and TIMPs are important regulators in many biological processes and diseases (Visse & Nagase 2003, W QIN, W LU and others. Melatonin inhibits MMP9 in HUVECs 150 Journal of Endocrinology (2012) 214, 145–153 www.endocrinology-journals.org Downloaded from Bioscientifica.com at 10/22/2018 10:55:46PM via free access
Gao et al. 2012). But the role of MMPs in HUVECs monolayer integrity is not yet well explored. To determine this query, we herein analyzed MMPs in HUVECs by gelatin zymography. It was revealed from the zymography analysis that MMP9 levels were increased in IL1β-treated HUVECs and melatonin pretreatment inhibited MMP9 activity. Meanwhile melatonin reduced MMP9 mRNA and protein levels and increased TIMP1 mRNA and protein levels by RT-PCR and western blot analysis. There was also evidence that melatonin showed a hepatoprotective role by the downregulation of MMP9 and the upregulation of TIMP1 expression in alcohol-induced liver injury (Mishra et al. 2011).

The endothelial barrier is an important structure that plays a vital role in the maintenance of vascular homeostasis (Thanabalasundaram et al. 2010). The fundamental basis for the barrier function is predominantly maintained by adherens junctions and tight junctions, which seal the paracellular pathway between adjacent endothelial cells (Bazzoni & Dejana 2004, Abbott et al. 2010). Main adherens junctions are composed of VE-cadherin, which is a special cadherin and appears in all endothelial cells of all types of vessels (Bazzoni & Dejana 2004). The main tight junction proteins are occludin, claudins, and ZO-1 (Thanabalasundaram et al. 2010). Therefore, to maintain and regulate the endothelium barrier function it is possible to involve the integrity of the adherens junctional protein VE-cadherin and the tight junctional protein occludin (Navaratna et al. 2007, Rangasamy et al. 2011). The integrity of junctional proteins between adjacent endothelial cells essentially indicates the extent of permeability of the endothelial cell barrier. Endothelial permeability changes are also associated with the activation of MMPs and the spatial redistribution of surface cadherin and occludin (Alexander & Elrod 2002). MMP9, secreted by endothelial cells, disrupted the blood–brain barrier and made leukocyte recruitment because it was able to degrade components of the basal membrane of cerebral vessels (Sulik & Chyczewski 2008). Endothelial MMP2, which is activated by breast cancer cell adhesion to VE cells, could degrade interendothelial junctional protein VE-cadherin and promote cancer cell transendothelial migration (Shen et al. 2010). Moreover, oxidants, inflammatory cytokines, and pharmacological agents can spur the activation of MMPs to destroy the adherens junctional protein VE-cadherin and the tight junctional protein occludin (Alexander & Elrod 2002, Navaratna et al. 2007). Our previous study suggested that IL1β increased the permeability of HUVECs monolayer by damaging adherens junctions and enlarging the junctions cleft width (Yuan et al. 2011). In this experiment, we found that MMP9 was elevated in IL1β-induced HUVECs and increased MMP9 enlarged the intercellular space by destroying cell junctional elements, VE-cadherin, and occludin. Meanwhile the permeability to Na-F was increased in this process.

Melatonin has pleiotropic bioactivities including as a highly effective antioxidant and free radical scavenger (Beyer et al. 1998, Gitto et al. 2009, Chahbouni et al. 2010, Paradies et al. 2010). Melatonin has protective effect against heart and brain ischemia/reperfusion injuries depending on its antioxidant and free radical scavenging properties (Paradies et al. 2010, Swarnakar et al. 2011). Melatonin has a regulatory role in MMP-mediated physiological processes and major human diseases. Recently, melatonin has been shown to prevent ethanol-induced gastric ulceration and liver injury in mice by downregulating MMP9 expression (Swarnakar et al. 2007, Mishra et al. 2011). In our study, melatonin pretreatment in IL1β-treated HUVECs inhibited the increase in MMP9, reversed the MMP9–damaged junctional proteins VE-cadherin and occludin, and attenuated the increase in HUVEC monolayer permeability to Na-F. Meanwhile, melatonin also increased the expression of the MMP9 inhibitor TIMP1 compared with the IL1β-treated group, which suggested that melatonin pretreatment could regulate the balance of MMP9 and TIMP1 to counteract the deleterious effects of MMP9. Moreover, Mostafa Mtairag et al. (2001) found that IL10 inhibited MMP9 activity and stimulated TIMP1 secretion in a dose-dependent manner in MM6/HUVEC cocultures.

Previous studies suggested that TNFα and/or IL1β could induce MMP9 expression via the NF-κB signaling pathway in different types of cells (Esteve et al. 2002, Xie et al. 2004, Liang et al. 2007). MMP9 is activated by reactive oxygen species and its expression is likely to be regulated by oxidative stress (Rajagopalan et al. 1996, Mori et al. 2004, Pustovrh et al. 2005). Studies in the literature indicate that melatonin significantly inhibits the translocation and binding of NF-κB to DNA in rat spleen and downregulates the expression of NF-κB/p65 in the pathophysiology of ischemia/reperfusion injury in a model of rat kidney transplantation (Chuang et al. 1996, Li et al. 2009). We observed that melatonin inhibited the nuclear translocation of p65 in IL1β-treated HUVECs and downregulated p65 and MMP9 proteins expression.

In summary, IL1β increases the expression and the activity of MMP9 is involved in disrupting adherens junctions and tight junctions, resulting in an increase in the permeability of endothelial cell monolayers. Melatonin pretreatment in HUVECs has a significant protection of HUVECs barrier via inhibiting MMP9 through the NF-κB pathway. We propose that this evidence may help to provide potential therapeutic actions of melatonin in the application of vascular system protection.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

W Q and W L planned and carried out experiments, analyzed data, and wrote the paper; H L, X Y, B L, and Q Z helped with experiments and analyzed data. R. X planned experiments and supervised all the work on this paper. All authors had final approval of the submitted and published versions.

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