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Original article

Melatonin has no direct effect on inflammatory gene expression in CML-HSA stimulated RAW264.7 cells

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Abstract

***Objective*:** Advanced glycation end products (AGEs), such as N^{ε}-(carboxymethyl)-lysine (CML), modified proteins activate macrophages and increase the production of inflammatory cytokines via receptors for advanced glycation end products (RAGE) mediated pathway. These compounds ultimately lead to the progress of acute and chronic inflammatory diseases like rheumatoid arthritis, knee osteoarthritis, locomotive syndrome and so on. Therefore, to suppress the upregulation of inflammatory gene expression is helpful way in both prevention and cure. Melatonin secretion declines with age, whereas AGE production and accumulation increases. In this study, we examined the effect of melatonin on CML-HSA (human serum albumin)-induced inflammatory gene expression in RAW264.7 cells.

***Methods*:** The mouse macrophage cell line RAW264.7 were treated with 10 and 0.1 μg/mL melatonin or 2 μL/mL dimethyl sulfoxide (DMSO) as the vehicle control for 24 hours, followed by stimulation by 0.5 μg/mL CML-HSA for 3 hours. TNFα protein content was analyzed by enzyme-linked immunosorbent assay (ELISA) and the inflammatory gene mRNA expression was measured by real-time polymerase chain reaction (RT-PCR). Cell viability was determined using a cell counting kit-8 (CCK-8) and lactate dehydrogenase (LDH) assay.

***Results*:** TNFα secretion was significantly elevated by CML-HSA stimulation for 3 hours ($p < 0.001$). Pretreatment with melatonin did not inhibit TNFα production, TNFα, RAGE, inducible nitric oxide synthase (iNOS), interleukin-1 beta (IL-1β), or IL-6 mRNA expression. Cell viability was not affected by the experimental conditions.

***Conclusion*:** These data suggested that melatonin does not have any inhibitory or stimulatory effect on CML-HSA-induced (glycative stress-induced) inflammatory gene expression in RAW264.7 cells.

***KEY WORDS*:** glycative stress, N^{ε}-(carboxymethyl)-lysine (CML), advanced glycation end products (AGEs), receptor for AGEs (RAGE), inflammation

Introduction

Advanced glycation end products (AGEs) are implicated in the pathogenesis of diabetic complications, cancer [1], retinal disease [2], cardiovascular disease [3], Alzheimer's disease [4], respiratory disorders [5], liver disease [6], chronic inflammatory diseases (atherosclerosis, asthma, arthritis), myocardial infarction, nephropathy, retinopathy, periodontitis and neuropathy [7] by AGE-RAGE (receptor for advanced glycation end products) interaction. AGEs also can interact with SR-A (macrophage scavenger receptor Type I and II), OST-48 (oligosaccharyl transferase-4) (AGE-R1), 80 K-H phosphoprotein (protein kinase C substrate) (AGE-R2), Galectin-3 (AGE-R3), LOX-1 (lectin-like oxidized low density lipoprotein receptor-1), and CD36.

N^{ε}-(carboxymethyl)-lysine (CML) is a major antigenic AGE which can activate RAGE-mediated reactive oxygen species (ROS), MAP kinase and nuclear factor-kappa B (NF-κB), ultimately leading to inflammatory cytokine formation [8]. Increased CML levels are observed in the cerebral blood vessels of both diabetic patients and in the streptozotocin-treated rat model of diabetes mellitus [9]. These evidences indicate that CML contributes to diabetes complications. CML has been identified in synovial tissue of rheumatoid arthritis (RA) patients, and presumably causes the perpetuation of inflammation in the joint [10]. In obese subjects, CML accumulation in adipose tissue, as evidenced by the decreased plasma levels of CML, plays an important role in adipose tissue inflammation [11].

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In our previous study, CML-HSA (0.5 to 2 μg/mL) was found to trigger tumor necrosis factor-alfa (TNFα; a major inflammatory cytokine) production in RAW264.7 cells; the TNFα level reached at peak after 3 hour of CML-HSA stimulation and declined thereafter [12]. In another study, we found that pretreatment with several kinds of plant extracts can significantly lower TNFα production in CML-HSA-induced RAW264.7 cells [13].

Melatonin (N-acetyl-5-methoxytryptamine) is a natural mammalian hormone synthesized by methylation of serotonin by SAMe (S-adenosyl methionine); it is mainly found in the pineal gland, but there is also some in retina, bone morrow, lymphocytes, and even in foods such as oats. Melatonin has a dual role as a potent anti-inflammatory [14-17] and an anti-oxidant [18]; it also has regulatory roles in sexual activity development [19], immunomodulation [20], and cardiovascular functions [21]. Further, it has been suggested to have anti-aging properties [22,23]. Melatonin secretion declines progressively with increasing age; menopause is time related with a substantial, sharp decrease in melatonin secretion [23-26]. Taken together, these facts that melatonin production drops significantly with age after puberty and that there is an age-related increase in CML accumulation both suggest a relation with many of the disturbances seen in the elderly.

Pathological inflammation is closely related to numerous different diseases, however the current anti-inflammatory drugs have significant side effects. Thus, it is important to identify new pharmacological approaches to reduce chronic inflammation without impairing the physiologic inflammatory responses. In this study, we have focused on the effect of melatonin on CML-HSA-induced inflammatory gene expression in RAW264.7 cells.

Materials and Methods

Materials

Melatonin was obtained from Sigma-Aldrich (cat # M5250, St. Louis, MO, USA) and dissolved into dimethyl sulfoxide (DMSO, Wako, Osaka, Japan). DMSO was also used as a vehicle control for treatments. CML-HSA was obtained from (CircuLex, Nagano, Japan). All other chemicals, of analytical grade, were obtained from Sigma-Aldrich or Wako.

Cell culture

Murine monocyte/macrophage RAW264.7 (ATCC® TIB-71™) cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Nichirei Biosciences, Tokyo, Japan), penicillin 100 units/mL, streptomycin 100 μg/mL and amphotericin B 25 μg/mL at 37 °C under the condition of 5% CO_2 [12].

Cell viability assessment

For determination of cell viability of RAW264.7 cells, WST-8 assay and lactate dehydrogenase (LDH) assay were used. WST-8 assay was performed using Cell Counting Kit-8 (CCK-8) according to the manufacturer's instructions (Dojindo, Kumamoto, Japan). LDH activity assay was performed as described by Saito *et al.* [27]. RAW264.7 cells were seeded at 2.5×10^4 cells/well in 96-well plates and incubated for 24 hours, then cells were treated with melatonin or DMSO for further 24 hours. For WST-8 assay, after 2 hours treatment of CML-HSA, 10 μL/well CCK-8 was added and incubated for another 1 hour. Absorbance at 450 nm was measured by a spectrophotometer [13].

For LDH assay, cultured media (50 μL) from each well was collected and used to determine the effect of CML-HSA and melatonin on cell cytotoxicity in RAW264.7 cells.

Isolation of total RNA and RT-PCR

RAW264.7 cells were seeded into 24-well plates at a density of 1×10^5 cells/well and incubated for 24 hours, and then, cells were pre-treated with melatonin or DMSO for further 24 hours. Then, cells were stimulated using 0.5 μg/mL CML-HSA for 3 hours.

Total RNA was extracted using Isogen II reagent (Nippon Gene, Toyama, Japan) according to the manufacturer's protocol. RNase-free DNase-treated total RNA (500 ng) was reverse-transcribed with PrimeScript™ RT Master Mix (Takara Bio Inc., Japan) using Applied Biosystems 2720 Thermal cycler.

RT-PCR was performed with a Thunderbird™ SYBR qPCR mix (Toyobo Co., Ltd., Osaka, Japan) according to manufacturer's protocol with gene-specific primers as listed in ***Table 1***. Briefly, the amplification reactions were carried out on a AB Applied Biosystems StepOnePlus Real-Time PCR system with an initial hold step (95°C for 1 min) and 40 cycles of PCR (95°C for 15 seconds, 60°C for 60 seconds), followed by dissociation curve. The comparative CT method was used to determine the amount of target, normalized to an endogenous reference (GAPDH; glyceraldehyde-3-phosphate dehydrogenase).

TNFα measurement by ELISA

Cells were pre-treated with melatonin or DMSO for 24 hours, and then, medium was replaced with DMEM containing 0.5 μg/mL CML-HSA for 3 hours. TNFα concentration in the cell culture medium was measured by ELISA (mouse TNFα ELISA kit, ab100747; Abcam, Cambridge, United Kingdom) as described by the manufacturer. Absorbance was measured at 450 nm by a spectrophotometer. TNFα concentrations were calculated using the standard curve prepared by the recombinant mouse TNFα with known concentrations (93.75 to 6,000 pg/mL).

Statistical analysis

Data were expressed as mean ± standard deviation (SD). All statistical analysis were performed using Tukey-Kramer test for intergroup comparison in all of the experiments. Differences were considered significant at a significance level of 5%.

Table 1. Primers for RT-PCR

Gene		Sequence (5 → 3)	
GAPDH			
	Forward	TGAAGGTCGGTGTGAACGGATTGGC	28)
	Reverse	CATGTAGGCCATGAGGTCCACCAC	
TNFα			
	Forward	ACCCTCACACTCAGATCATCTTC	29)
	Reverse	TGGTGGTTTGCTACGACGT	
IL-6			
	Forward	ACAACCACGGCCTTCCCTACTT	29)
	Reverse	CACGATTTCCCAGAGAACATGTG	
iNOS			
	Forward	CCAAGCCCTCACCTACTTCC	29)
	Reverse	CTCTGAGGGCTGACACAAGG	
IL-1β			
	Forward	TGTAATGAAAGACGGCACACC	30)
	Reverse	TCTTCTTTGGGTATTGCTTGG	
RAGE			
	Forward	ACTACCGAGTCCGAGTCTACC	31)
	Reverse	GTAGCTTCCCTCAGACACACA	

RT-PCR, real-time polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNF, tumor necrosis factor; IL, interleukin; iNOS, inducible nitric oxide synthase; RAGE, receptor for AGEs; AGEs, advanced glycation end products.

Results

Cell viability assessment

First, we analyzed the effect of melatonin and CML-HSA on cell viability in RAW264.7 cells. WST-8 assay was performed to measure the percentage of viable cells in response to melatonin and CML-HSA treatment for 24 hours (***Fig. 1A***). Also conditioned medium was collected to measure LDH released into the media from damaged cells (***Fig. 1B***). Either 10 μg/mL melatonin or 0.5 μg/mL CML-HSA did not affect cell viability in RAW264.7 cells.

Effect of melatonin on CML-HSA-induced inflammatory gene expression

Cells were pre-treated with melatonin as 10 & 0.1 μg/mL or 2 μL/mL DMSO (vehicle control) for 24 hours, followed by stimulation by 0.5 μg/mL CML-HSA for 3 hours. RNA was extracted and performed RT-PCR analysis to measure mRNA expression of TNFα and condition medium were used for ELISA to determine secreted TNFα. CML-HSA induced both mRNA levels (***Fig. 2A***) and secreted protein levels into culture medium (***Fig. 2B***). In this condition, pre-treatment of 10 μg/mL melatonin lowered CML-HSA-induced TNFα mRNA expression compared to the vehicle control (***Fig. 2A***), however, the secreted protein levels of TNFα did not altered by melatonin (***Fig. 2B***). To further investigation of inflammation responses, we performed RT-PCR analysis against inflammatory genes such as iNOS, IL-1β, IL-6. There was a trend of inducing inflammatory genes by CML-HSA, however, these are not significant and not altered by melatonin (***Fig. 3***).

Inhibition of basal expression of inflammatory gene by melatonin

In ***Figs. 2*** and ***3***, melatonin did not show significant inhibition of inflammatory genes in response to CML-HSA, we further investigated that the effect of melatonin on basal expression levels of inflammatory genes in RAW264.7 cells. In case of without CML-HSA treatment, melatonin showed a trend to decrease basal mRNA levels of TNFα and IL-6 expression (***Figs. 4A*** and ***4B***) and significantly inhibited iNOS and IL-1β (***Figs. 4C*** and ***4D***).

Neither CML-HSA nor melatonin did alter expression of RAGE mRNA in RAW264.7 cells

RAGE is a well known receptor for AGEs. In this study, we examined that the effect of CML-HSA and melatonin on RAGE expression. As shown in ***Figs. 5A*** and ***5B***, neither CML-HSA nor melatonin did affect RAGE expression.

Discussion

AGEs can disrupt or change cellular functions by (a) the formation of cross-links between key molecules, permanently altering cellular structure; and (b) activation of AGEs receptors which causes upregulation of the transcription factor NF-κB and its target genes [32]. The engagement of AGEs with RAGE triggers NADPH-oxidase, increasing intracellular oxidative stress, evokes signaling pathways such as protein kinase C, mitogen-activated protein kinase (MAPK) and the extracellular signal-regulated kinase; it activates proinflammatory transcription factor NF-κB and activator protein 1 (AP-1), and subsequently induces thrombogenic, fibrogenic, adhesive, chemoattractive and proinflammatory [*i.e.* TNFα, IL-1, IL-6, matrix metalloproteinase (MMP), vascular endothelial growth factor (VEGF)] gene expression [12,33-36]. IL-1 (α and β), IL-6 and TNF (α and β) induce macrophage differentiation into osteoclast-like multinucleated cells and bone resorption by increasing the bone-resorbing activity of formed osteoclasts *in vitro* and *in vivo* [37-42].

TNFα was also reported to enhance the receptor activator of nuclear factor kappa-B ligand (RANKL) signaling by inducing the formation of osteoclastic cells in the presence of low concentrations of RANKL from bone marrow macrophages *in vitro* [40,42], indicating that TNFα is a crucial differentiation factor for osteoclasts. TNFα and M-CSF (macrophage colony-stimulating factor) play an important role in local osteolysis in chronic inflammatory diseases. TNFα strongly induces differentiation of M-CSF-dependent bone marrow macrophage (MDBM) cells into mature osteoclasts, TRAP-positive multinucleated cells (MNCs) in the presence of M-CSF, and in the presence or absence of RANKL [42]. Diseases like rheumatoid arthritis, knee osteoarthritis, locomotive syndrome, aseptic loosening, and periodontal diseases have been associated with the accumulation of pro-inflammatory cytokines, TNFαand/or IL-1 and IL-6, which likely facilitates local bone destruction by stimulating osteoclast differentiation and activation [43-45]. Therefore, proinflammatory cytokine production is closely related to bone diseases.

Proteins are usually glycated through their lysine residues and in humans, histones in the cell nucleus are the richest in lysine, and therefore the formation and accumulation of glycated protein CML is very common [46]. Elevated levels of CML and pentosidine in serum may be a cause of bone loss due to disturbed bone remodeling in osteoporosis patients [47]. RAGE, when binding with AGEs, contributes to age- and diabetes-related chronic inflammatory diseases such as atherosclerosis, asthma, rheumatoid arthritis, knee osteoarthritis, locomotive syndrome, osteoporosis, myocardial infarction, nephropathy, retinopathy, periodontitis and neuropathy [7]. Thus AGEs induce inflammatory gene expression and these inflammatory cytokines subsequently lead to destructive bone diseases. Thus, the inhibition of inflammatory gene expression is of great importance in chronic inflammatory and destructive bone diseases.

Inducible nitric oxide synthase (iNOS) is a key enzyme for generating NO (a free radical with an unpaired electron) as an immune defense mechanism by activated macrophages. It is the proximate cause of septic shock and may function in autoimmune disease. IRF1 and NF-κB-dependent activation of the inducible NOS promoter supports an inflammation-mediated stimulation of this transcript. iNOS produces large quantities of NO upon stimulation, such as by proinflammatory cytokines (*e.g.* TNFα, IL-1 and interferon gamma) [48]. Pathologic generation of nitric oxide through increased iNOS production may decrease tubal ciliary beats and smooth muscle contractions and thus affect embryo transport, which may consequently result in ectopic pregnancy [49,50]. Therefore,

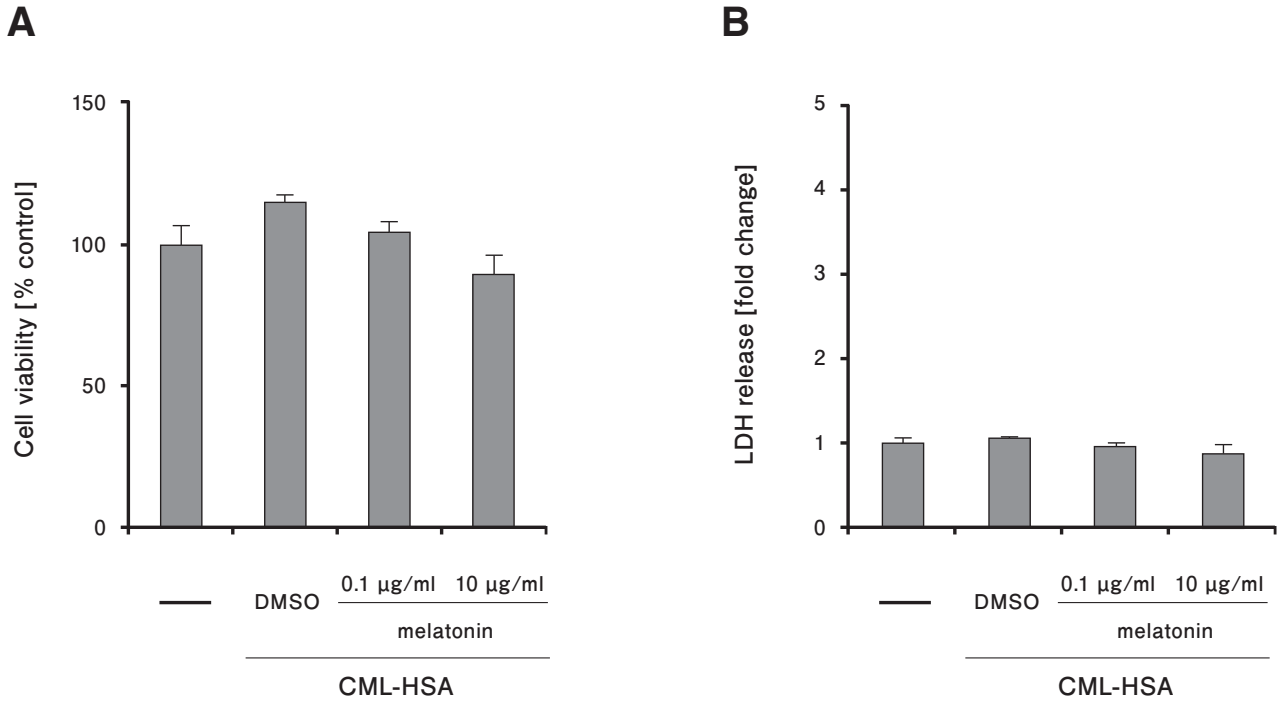

Fig. 1. **Effect of CML-HSA and melatonin on cell viability in RAW264.7 cells.**

Cells were pre-incubated with melatonin or DMSO for 24 hours, then stimulated with CML-HSA for further 3 hours. ***A***. WST-8 assay was performed to determine cell viability. ***B***. Condition medium was used for LDH assay to assess dead cells. All data were shown as the mean ±standard deviation (n = 3) of the ratios against no addition. CML, carboxymethyl-lysine; HSA, human serum albumin; DMSO, dimethyl sulfoxide; LDH, lactate dehydrogenase.

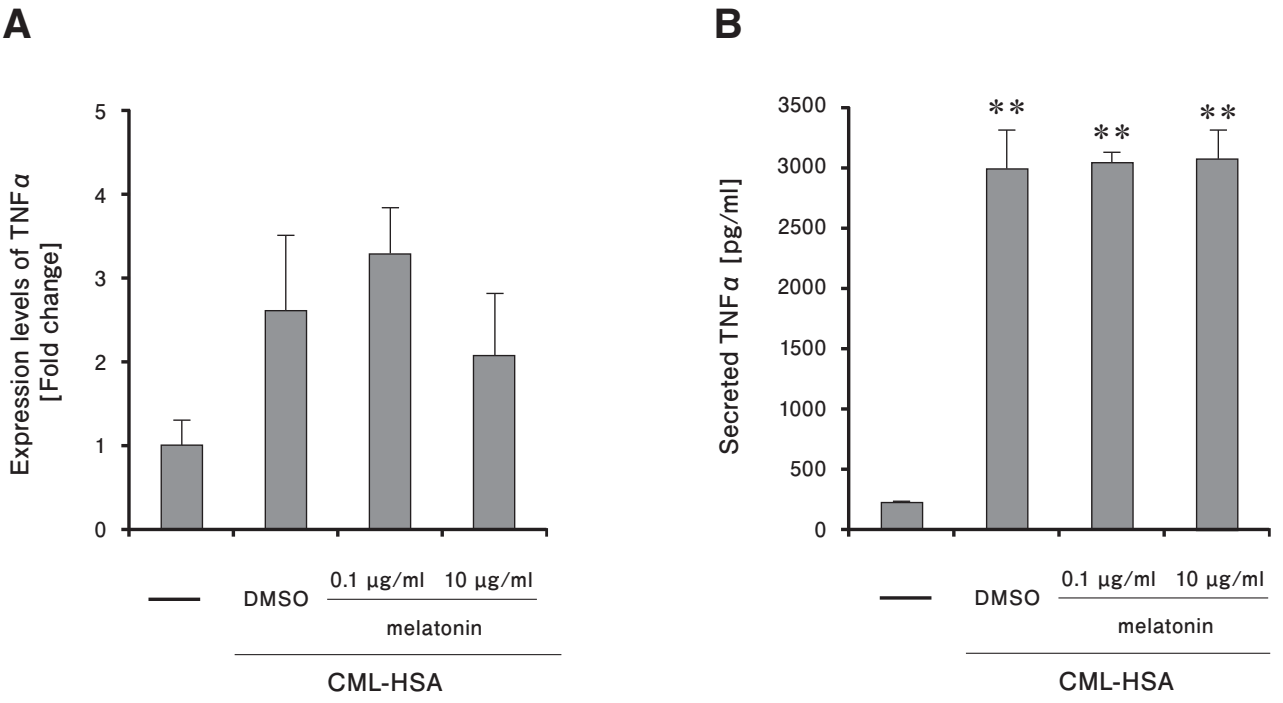

Fig. 2. **Effect of melatonin on CML-HSA induced TNFα gene expression.**

Cells were pre-incubated with melatonin or DMSO for 24 hours, then stimulated with CML-HSA for further 3 hours. ***A***. TNFα mRNA level was examined by RT-PCR. ***B***. Secreted TNFα was determined using condition medium by ELISA. All data obtained were normalized by *GAPDH* value and were shown as the mean ± standard deviation (n = 3) of the ratios against no addition. ** $p < 0.01$ *vs.* no addition by Tukey-Kramer test. CML, carboxymethyl-lysine; HSA, human serum albumin; TNF, tumor necrosis factor; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

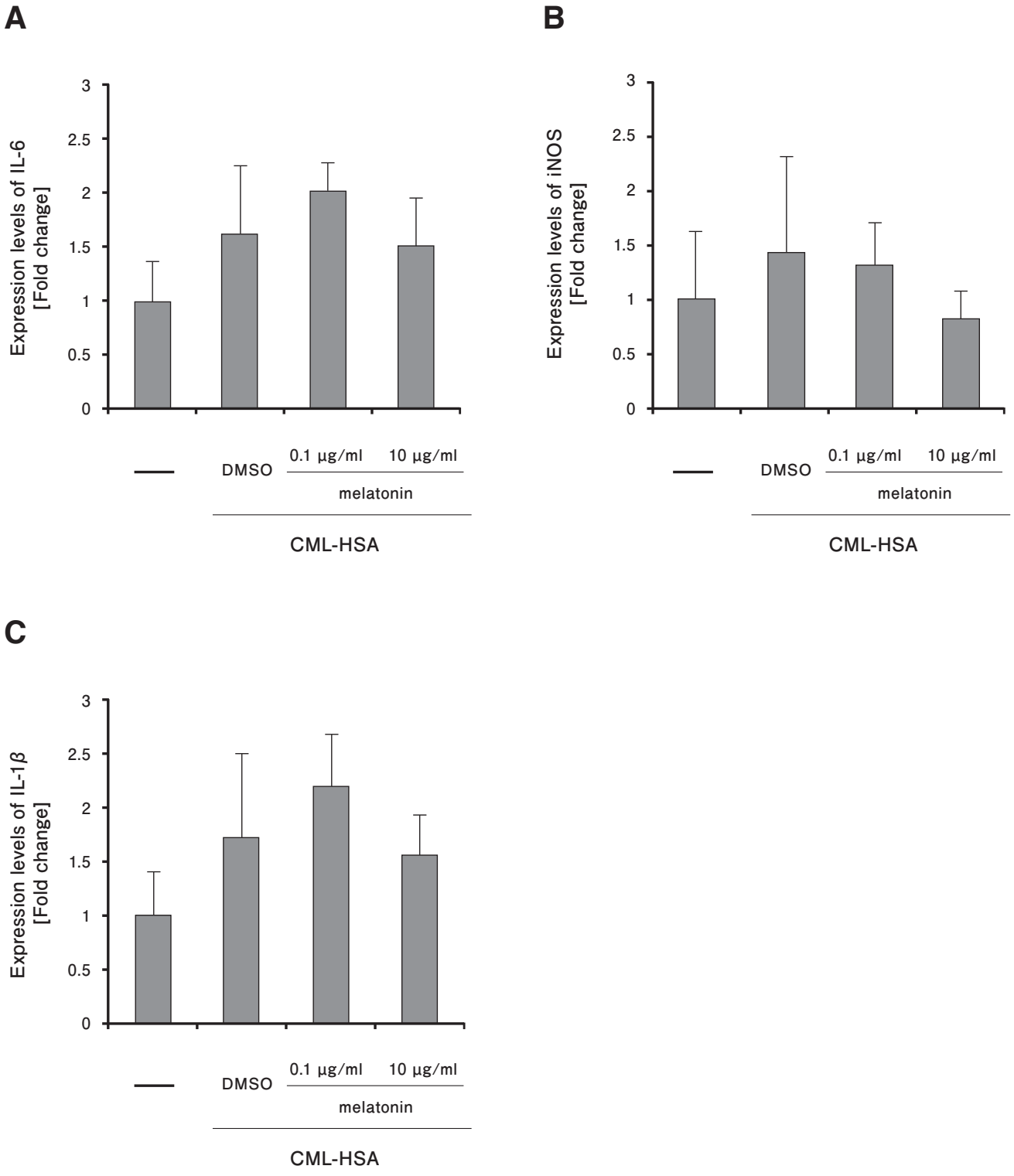

Fig. 3. **Effect of melatonin on CML-HSA-induced inflammatory genes expression in RAW264.7 cells.**
Cells were pre-incubated with melatonin or DMSO for 24 hours, then stimulated with CML-HSA for further 3 hours. RT-PCR analyses were performed for ***A***. IL-6, ***B***. iNOS and ***C***. IL-1β. All data obtained were normalized by *GAPDH* value and were shown as the mean ± standard deviation (n = 3) of the ratios against no addition. CML, carboxymethyl-lysine; HSA, human serum albumin; DMSO, dimethyl sulfoxide; IL, interleukin ; iNOS, inducible nitric oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

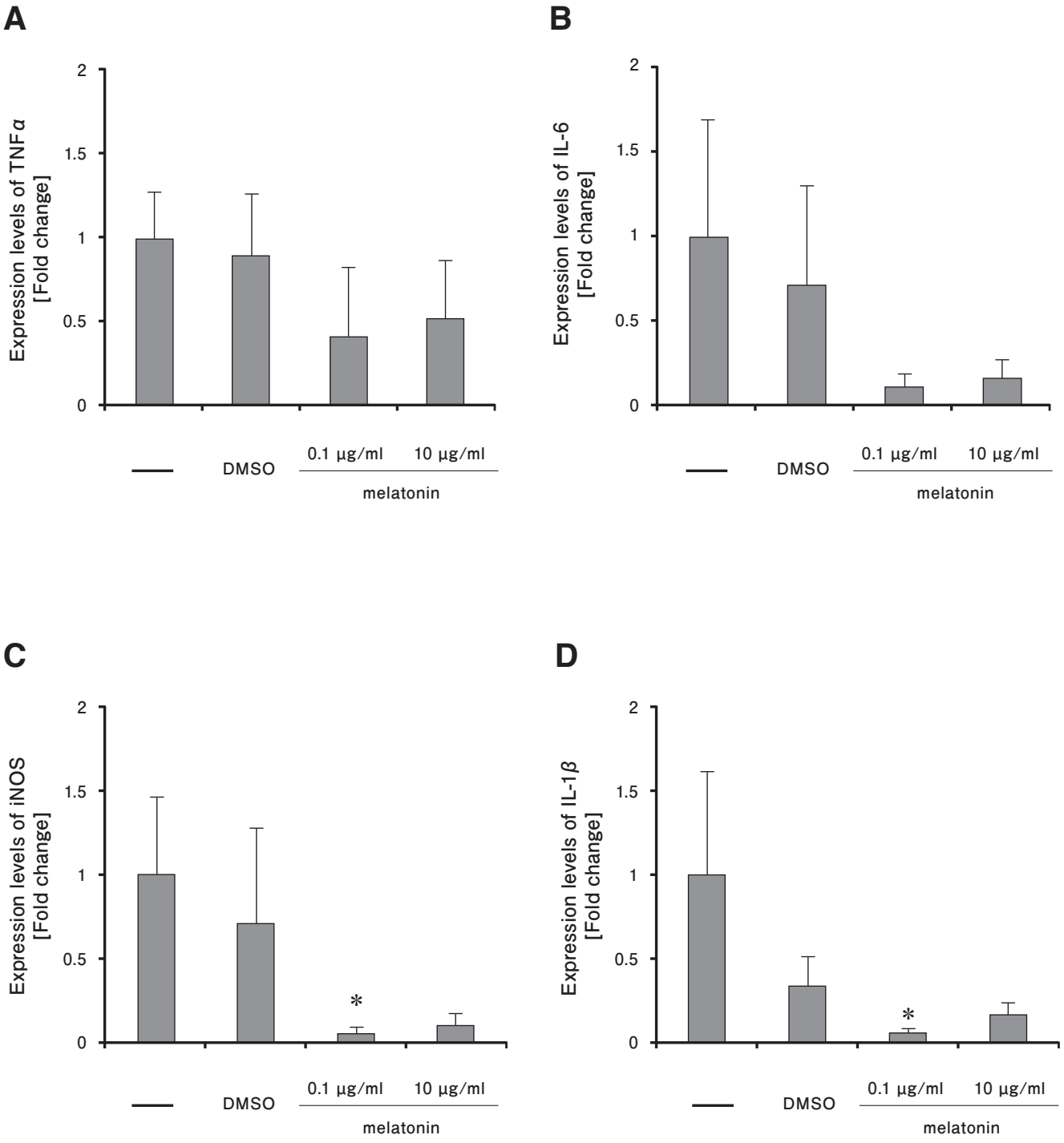

Fig. 4. **Effect of melatonin on inflammatory genes expression in RAW264.7 cells.**

Cells were incubated with melatonin or DMSO for 24 hours, then RNA was extracted and performed RT-PCR. ***A***. TNFα, ***B***. IL-6, ***C***. iNOS and *D*. IL-1β. All data obtained were normalized by *GAPDH* value and were shown as the mean ± standard deviation (n = 3) of the ratios against no addition. * $p < 0.05$ *vs*. no addition by Tukey-Kramer test. DMSO, dimethyl sulfoxide; TNF, tumor necrosis factor; IL, interleukin ; iNOS, inducible nitric oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase .

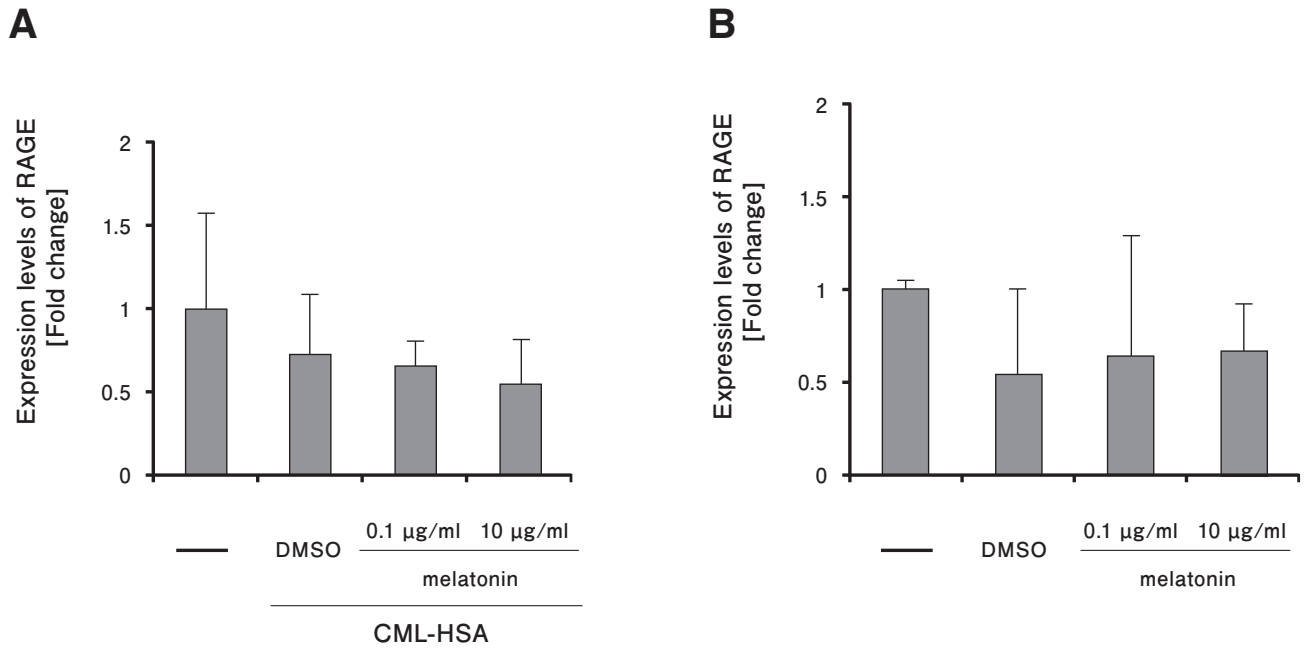

Fig. 5. **Effect of melatonin and CML-HSA on RAGE expression.**

Cells were incubated with melatonin or DMSO for 24 hours (***B***), then stimulated CML-HSA for further 3 hours (***A***). RNA was extracted and performed RT-PCR against RAGE. All data obtained were normalized by *GAPDH* value and were shown as the mean ± standard deviation (n = 3) of the ratios against no addition. CML, carboxymethyl-lysine; HSA, human serum albumin; RAGE, receptor for advanced glycation end products; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase .

inhibition of TNFα, IL-1β, IL-6, iNOS, and RAGE expression pathway is of great importance to control chronic inflammatory diseases. Anti-inflammatory medications such as steroids have strong anti-inflammatory effects but can cause undesirable side effects like adrenal suppression, cataracts, and negative effects on bone metabolism. Nonsteroidal anti-inflammatory drugs (NSAIDs) can cause alterations in renal function, hepatic injury, and induce gastric ulcer [51,52]. Development of anti-inflammatory drugs with a high efficacy and few side effects is necessary for the treatment of acute and chronic inflammatory diseases including rheumatoid arthritis, atherosclerosis, Alzheimer's disease, knee osteoarthritis, and locomotive syndrome [53].

Melatonin (N-acetyl-5-methoxytriptamine), a hormone secreted by the pineal gland that is involved in the mediation of circadian rhythms, has anti-inflammatory effects produced via decreased levels of prostaglandins, NO, and TNFα both *in vitro* and *in vivo* [23,54-58]. Melatonin can also reduce the expression of COX-2 and iNOS genes [56] and has minimal side effects in humans [59]. Melatonin and its derivatives, benzoyl-melatonin and acetyl-melatonin, inhibited nitric oxide NO and prostaglandin E2 production in lipopolysaccharide (LPS)-stimulated RAW264.7 cells in a dose-dependent manner with half maximum inhibition concentration (IC_{50}) values lower than those of melatonin [60].

In the present study, we investigated that the effect of melatonin on inflammatory genes in RAW264.7 cells with or without glycated protein such as CML-HSA. CML-HSA significantly stimulated RAW264.7 cells to produce TNFα in all of our experimental conditions, but melatonin did not change the TNFα mRNA level or protein content significantly compared to the vehicle control group (DMSO 2 μL/mL) in 0.5 μg/mL CML-HSA stimulated RAW264.7 cells. Though melatonin at 10 μg/mL lowered TNFα mRNA expression slightly compared to the vehicle control group, the secreted protein levels were the same (***Fig. 2***). As shown in ***Fig. 3***, melatonin did not change the iNOS, IL-1β, or IL-6 mRNA level significantly compared to the vehicle control group (DMSO 2 μL/mL) in response to CML-HSA. As while, melatonin itself decreased basal mRNA expression levels of iNOS and IL-1β (***Figs. 4C*** and ***4D***). None of the experimental conditions significantly changed cell viability (***Figs. 1*** and ***2***) and expression of RAGE (***Fig. 5***) in RAW264.7 cells.

Melatonin prevented inflammatory nitric oxide NO and prostaglandin E2 production in LPS-stimulated RAW264.7 cells in a dose-dependent manner with IC_{50} values 1,500 μM, and 840 μM, respectively [60] which is much higher than the peak value of melatonin secretion in humans 0.516~0.775 nM [61,62]. In our experimental conditions we did not find any significant inhibition on inflammatory TNFα, RAGE, iNOS, IL-1β, IL-6 mRNA or TNFα protein expression by using 0.1~10 μg/mL melatonin (approximately 0.43~43.05 μM), 55,548 times higher concentration than the peak value in humans. As melatonin is a sleep causing hormone and such a high concentration cannot reduce tested inflammatory gene expression by CML-HSA induced RAW264.7 cells likely indicates that melatonin has no direct effect on CML-mediated inflammatory gene expression by macrophage

cells. Therefore, prevention and blockage of CML-modified protein production, that is glycation, is of great importance for the prevention of AGEs-mediated health complications. Melatonin derivatives, benzoyl-melatonin and acetyl-melatonin have much higher anti-inflammatory effects [60]. However, further studies will be necessary on the effects of melatonin derivatives on CML-HSA-induced inflammatory gene expression. Also animal studies are necessary as the immune system consists of many types of cellular interactions.

In our previous study demonstrated that LPS stimulates much higher inflammatory gene expression than CML in the same RAW264.7 cell culture [12]. Phiphatwatcharaded C *et al*. reported that melatonin can prevent inflammatory gene expression induced by LPS [60], but not by CML. These data indicate that CML-HSA might activate different pathway from LPS activates. Therefore, further studies are necessary to elucidate the pathways in order to determine effective blockers of CML-induced inflammatory gene expression. As another study showed that several foods have potential anti-glycative and anti-inflammatory activity, some plant materials can be used in the future to prevent CML production in the human body and thereby lessen inflammatory gene expression.

Conclusion

The present study showed that melatonin pre-treatment does not have any inhibitory or stimulatory effect on CML-HSA-induced TNFα mRNA expression and protein secretion in RAW264.7 cells.

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Conflict of Interest Statement

The authors state that the performance of this study entailed no issues representing a conflict of interest.

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