Inhibition of protein kinase CK2 facilitates cellular senescence by inhibiting the expression of HO-1 in articular chondrocytes

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Abstract. Protein kinase casein kinase 2 (CK2) is important in the regulation of cell proliferation and death, even under pathological conditions. Previously, we reported that CK2 regulates the expression of heme oxygenase-1 (HO-1) in stress-induced chondrocytes. In the present study, it was shown that CK2 is involved in the dedifferentiation and cellular senescence of chondrocytes. Treatment of primary articular chondrocytes with CK2 inhibitors, 4,5,6,7-terabromo-2-azabenzimidazole (TBB) or 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside (DRB), induced an increase in senescence-associated β-galactosidase (SA-β-gal) staining. In addition, TBB reduced the expression of type II collagen and stimulated the accumulation of β-catenin, phenotypic markers of chondrocyte differentiation and dedifferentiation, respectively. It was also observed that the abrogation of CK2 activity by CK2 small interfering RNA induced phenotypes of chondrocyte senescence. The association between HO-1 and cellular senescence was also examined in CK2 inhibitor-treated chondrocytes. Pretreatment with 3-morpholinosydnonimine hydrochloride, an inducer of the HO-1 expression, or overexpression of the HO-1 gene significantly delayed chondrocyte senescence. These results show that CK2 is associated with chondrocyte differentiation and cellular senescence and that this is due to regulation of the expression of HO-1. Furthermore, the findings suggest that CK2 is crucial as an anti-aging factor during chondrocyte senescence.

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Abbreviations: CK2, protein kinase casein kinase 2; TBB, 4,5,6,7-terabromo-2-azabenzimidazole; DRB, 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside; SA-β-gal, senescence-associated β-galactosidase; HO-1, heme oxygenase-1; SIN-1, 3-morpholinosydnonimine hydrochloride; ECM, extracellular matrix; ROS, reactive oxygen species; OA, osteoarthritis

Key words: protein kinase casein kinase 2, chondrocytes, senescence-associated β-galactosidase, cellular senescence, heme oxygenase-1

Introduction

The degradation of articular cartilage is a major characteristic of several arthritic diseases, including osteoarthritis (OA) and rheumatoid arthritis (RA), which eventually result in joint destruction (1,2). The biosynthetic activities of chondrocytes contribute to the stability of articular cartilage (3-5), which contains extracellular matrix (ECM) molecules that confer mechanical elasticity. By contrast, chondrocyte dysfunction enhances cartilage ECM degradation and cartilage degeneration (6,7). Chondrocyte senescence is also important in articular cartilage degeneration (8), as it is associated with declines in chondrocyte numbers and contributes to the development of arthritic diseases (9,10). It has been established that senescent chondrocytes accumulate in the articular cartilages of patients with arthritic diseases (11-13), however, the factors that govern chondrocyte decisions regarding senescence remain to be fully elucidated.

Protein kinase casein kinase 2 (CK2) is a constitutively active and highly conserved serine/threonine protein kinase consisting of two catalytic (α and/or α') and two regulatory (β) subunits (14). CK2 is ubiquitously expressed in subcellular compartments in all eukaryotes and phosphorylates over 300 substrates (15), and regulates several different metabolic events as a result. Several studies have demonstrated the links between CK2 and cell growth (16,17), transformation (18) and apoptosis (19), and between CK2 and inflammatory diseases (16,20). However, despite research efforts on various aspects of CK2, its function in chondrocytes is only partly understood. Previously, CK2 was reported to be involved in the regulation of rat articular chondrocyte apoptosis by phosphorylating certain apoptosis-related factors (21). In another study, it was suggested that inhibition of the activity of CK2 facilitates tumor necrosis factor-mediated chondrocyte death through apoptosis, and that the activity of CK2 is downregulated in the chondrocytes of aged articular cartilage (22).

In our previous study, it was shown that CK2 is associated with the expression of heme oxygenase-1 (HO-1) in articular

chondrocytes (23). In the present study, it was observed that inhibiting the activity of CK2 induces the senescence of primary articular chondrocytes. In addition, it was found that CK2 inhibition-mediated senescence is associated with regulation of the expression of HO-1 in chondrocytes.

Materials and methods

Reagents. The 4,5,6,7-terabromo-2-azabenzimidazole (TBB), 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside (DRB), 3-morpholinosydnonimine hydrochloride (SIN-1), protease inhibitor cocktail, trypan blue solution (0.4%), 5-bromo-4-chloroindol-3-indolyl β-D-galactopyranoside, potassium ferrocyanide, and potassium ferricyanide were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and other culture reagents were purchased from GE Healthcare Life Sciences (Logan, UT, USA). Anti-CK2 (sc-373894), HO-1 (sc-10789), type II collagen (sc-52658), β-catenin (sc-7199), and β-actin (sc-1616-R) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Secondary horseradish peroxidase (HRP)-conjugated antibodies (anti-rabbit, NA934; anti-mouse, NA931) and the enhanced chemiluminescence (ECL) western blotting kit were obtained from GE Healthcare Life Sciences.

Animals and cell culture of articular chondrocytes. Five-week-old female Sprague-Dawley rats were purchased from Samtako Bio Korea, Co. (Osan, Korea). All animal experiments and protocols were approved by the Pusan National University Institutional Animal Care and Use Committee (Miryang, Korea) and performed in accordance with the institutional and national guidelines for the care and use of laboratory animals. The knee joint cartilages were collected shortly upon arrival, however if necessary, the rats were housed under controlled conditions at 23±2˚C and 50±10% humidity on a 12 h light/dark cycle and had free access to a standard chow diet and water.

A total of 30 rats were used in the present study and their weight range was 110-130 g. To obtain primary articular chondrocytes, the knee joint cartilages from three rats per experiment were cut into ~1-mm^3 sections, and the samples were incubated for 1 h at 37˚C with 0.2% type II collagenase in DMEM. Primary chondrocytes were collected by centrifugation (300 x g; 5 min; room temperature) and resuspended in DMEM supplemented with 10% (v/v) heat-inactivated FBS and antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin) at 37˚C in a 5% CO_2/95% air atmosphere. The cells were then plated on culture dishes at 5x10^4 cells/cm^2 and cultured until confluent for ~4-5 days (medium was replaced every 2 days during culture). Following subculture (the cells were designated as passage 1, which was used for all experiments), cells were plated at a density of 1x10^5 cells/cm^2 and cultured for 24 h in 37˚C incubator under a humidified atmosphere conditions with 5% CO_2. Then, the cells were treated with various concentrations (2, 10, and 50 μM) of TBB or DRB in the presence or absence of SIN-1 for 48 h. The cell viabilities were determined using a trypan blue exclusion assay with a hemocytometer.

Senescence-associated β-galactosidase (SA-β-gal) staining assay. The cells were washed in phosphate-buffered saline (PBS), fixed for 5 min at room temperature in 0.2% glutaraldehyde/2% formaldehyde, washed with PBS, and incubated with SA-β-gal staining solution [1 mg/ml 5-bromo-4-chloro-indol-3-indolyl β-D-galactopyranoside, 40 mM citrate/sodium phosphate buffer (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM $MgCl_2$] for 12 h at 37˚C. The percentages of senescent cells were determined by counting the numbers of blue-stained cells per 200 cells in randomly selected areas at x20 magnification under an optical microscope.

Western blot analysis. The cells were lysed in ice-cold RIPA buffer with protease inhibitors, and protein concentrations in the whole cell lysates were determined using a colorimetric method with bicinchoninic acid protein dye reagent (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Subsequently, the proteins (20 μg) were loaded onto 12% SDS-polyacrylamide gels, electrophoresed, and transferred onto nitrocellulose membranes using an electroblotting apparatus (Bio-Rad Laboratories, Inc., Richmond, CA, USA). Following blocking with 5% skim milk in TBS-T, the membranes were incubated with primary antibodies targeting CK2α (1:250), HO-1 (1:250), type II collagen (1:250), β-catenin (1:250), and β-actin (1:500) at 4˚C overnight, washed with TBS-T, incubated with HRP-conjugated secondary antibodies (1:2,000) at room temperature for 2 h, and developed using the ECL detection system. The blots were visualized and quantified using a LAS-3000 Luminescent Image Analyzer (FujiFilm, Tokyo, Japan). β-actin was used as an internal loading control.

Measurement of CK2 kinase activity. The activity of CK2 was measured in cell lysates using a CK2 kinase assay kit (CycLex Co., Ltd., Ina, Japan). Briefly, the purified recombinant CK2 (for standard curve) and cell lysates (2x10^5 cells each) from CK2α small interfering (si)RNA or SCR-transfected cells were added to the wells of a CK2 substrate (recombinant p53)-precoated plate. Following the addition of kinase reaction buffer, the wells were washed and incubated with HRP-conjugated detection antibody at room temperature for 30 min. The phosphorylation activities were assessed by adding tetramethylbenzidine and measuring the absorbances at 450 nm. All quantifications were performed in triplicate.

Transfection of CK2α siRNA. To knock down CK2 gene expression, a 21-nucleotide RNA duplex containing 3'-dTdT overhangs (GE Healthcare Dharmacon, Inc., Lafayette, CO, USA) was used to target rat CK2α mRNA (sequence, 5'-CTGGGTGGGTGTCTCATTCAA-3'). At 24 h after plating, the chondrocytes at ~70% confluence were transfected with 30 nM of CK2α or negative control siRNA in wells using DharmaFECT® Duo transfection reagent, according to the manufacturer's protocol.

Preparation of HO-1 expression vector and transfection. The pcDNA3 mammalian cell expression vector containing full-length rat HO-1 cDNA was used as previously

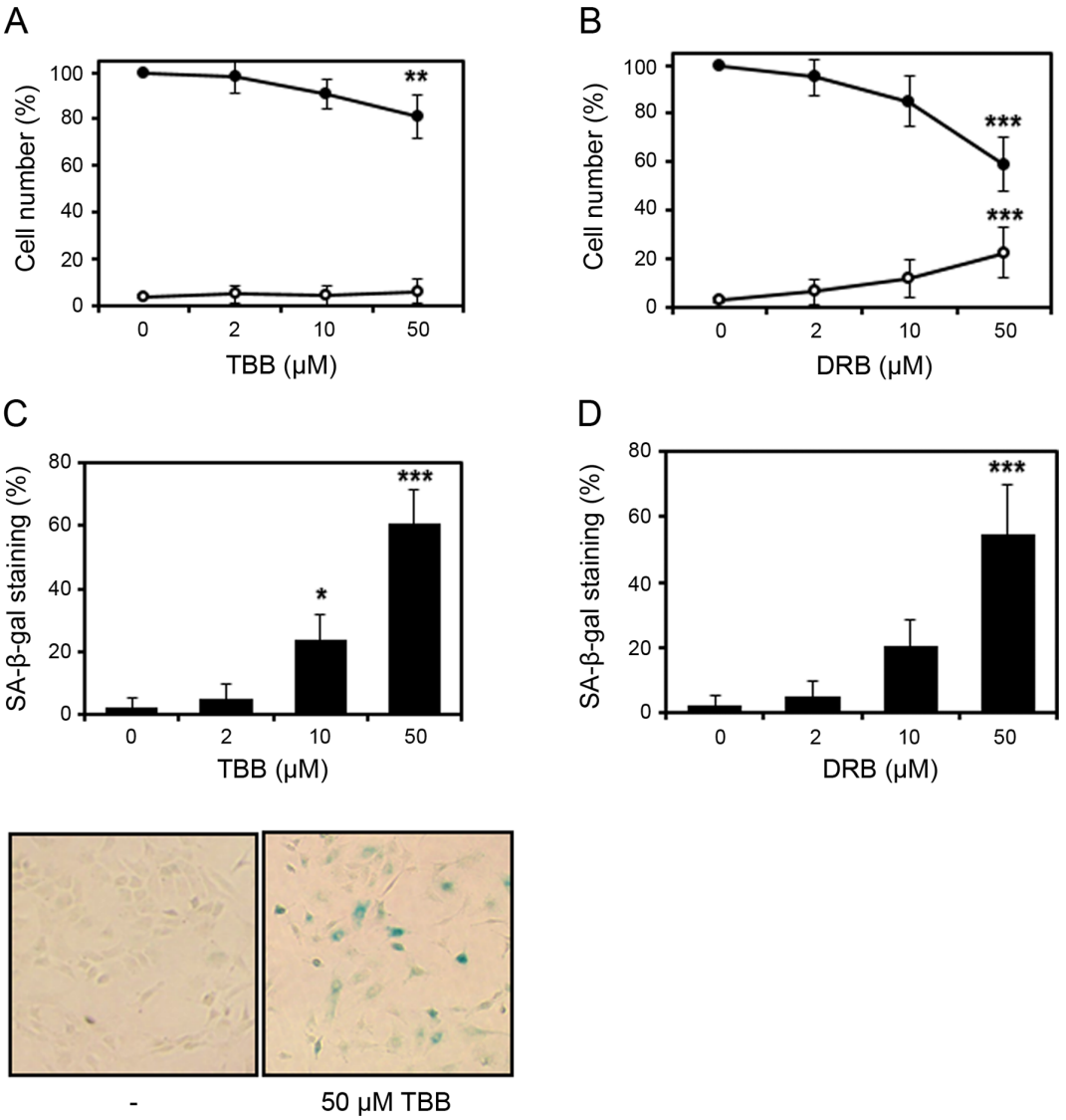

Figure 1. Effect of CK2 inhibitors on chondrocyte senescence. Primary rat articular chondrocytes were treated with different concentrations (2, 10 and 50 μM) of TBB or DRB for 48 h. Cells treated with (A) TBB and (B) DRB were assessed for viability using the trypan blue dye exclusion method (closed circles represent live cells, open circles represent dead cells). Values are shown as the percentages of control cells. Chondrocyte senescence was measured in cells treated with (C) TBB and (D) DRB using the SA-β-gal assay. Percentages of SA-β-gal-positive cells were calculated from numbers of blue-stained cells per 200 cells in randomly selected areas. Representative images were captured at x20 magnification. The P-value was calculated by one-way analysis of variance followed by Dunnett's post hoc test. *P<0.05, **P<0.01 and ***P<0.001 vs. 0 μM of TBB or DRB. CK2, protein kinase casein kinase 2; TBB, 4,5,6,7-terabromo-2-azabenzimidazole; DRB, 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside; SA-β-gal, senescence-associated β-galactosidase.

described (24). The chondrocytes (1x10^5 cells/cm^2) were transfected with 10 μg of the HO-1 construct or control plasmid complexed with Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), incubated for 24 h in a 37˚C incubator, placed in fresh medium, and then treated with TBB. The cells were also analyzed to monitor the expression level of HO-1.

Statistical analysis. All experiments were repeated at least three times. Student's t-test (two-tailed) or one-way analysis of variance were used to analyze the data by using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). Dunnett's or Tukey's honest significant difference post hoc tests were used. The results are presented as the mean ± standard deviation, and P<0.05 was considered to indicate a statistically significant difference.

Results

TBB and DRB induces primary rat chondrocyte senescence. To determine whether the activity of CK2 is associated with cellular senescence in chondrocytes, the present study first examined the effect of TBB and DRB (pharmacological inhibitors of CK2) in primary rat chondrocytes. Isolated cells were treated with various concentrations (2, 10 and 50 μM) of TBB or DRB for 48 h and then subjected to trypan blue dye exclusion assay and SA-β-gal staining assay. As shown in Fig. 1, TBB marginally reduced cell proliferation without affecting cell viability and significantly increased SA-β-gal staining compared with the untreated controls. The DRB-treated chondrocytes exhibited significant decreases in cell proliferation, although DRB also increased cellular senescence of chondrocytes (Fig. 1A-D). Therefore, TBB was only used as

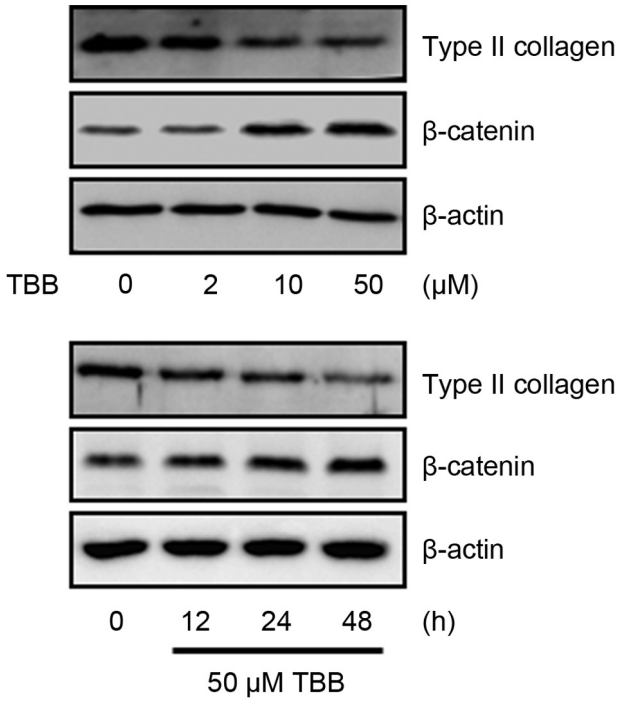

Figure 2. Effect of TBB on the expression of chondrocyte phenotypic markers. Primary chondrocytes were treated with different concentrations of TBB for 48 h and for different time intervals, as indicated. Whole cell lysates were analyzed by western blotting using specific antibodies against type II collagen and β-catenin. β-actin was used as an internal control. TBB, 4,5,6,7-terabromo-2-azabenzimidazole.

a specific inhibitor to downregulate the activity of CK2 in further experiments. Subsequently, the effect of TBB on the expression of phenotypic markers were examined, including type II collagen and β-catenin, which regulate chondrocyte functions. The primary chondrocytes were treated with different concentrations (2, 10 and 50 μM) of TBB and for different time intervals (12, 24 or 48 h). As shown in Fig. 2, TBB significantly suppressed the expression of type II collagen and enhanced the expression of β-catenin. These results show that TBB induced changes in the expression of phenotypic markers of the differentiation and dedifferentiation of primary chondrocytes.

Specific inhibition of the activity of CK2 enhances chondrocyte senescence. To confirm that the activity of CK2 is associated with chondrocyte senescence, CK2 was knocked down by transfecting chondrocytes with a CK2 siRNA duplex or a non-specific control for 24 h and then incubating the cells for a further 48 h. Transfection was found to effectively inhibit CK2 enzyme activity and to significantly reduce the protein levels of CK2 in chondrocytes (Fig. 3A and B). Transfection with control siRNA did not affect SA-β-gal staining significantly compared with the non-transfected controls. CK2 knockdown significantly increased SA-β-gal staining (Fig. 3C), and reduced the protein levels of type II collagen and enhanced β-catenin in chondrocytes. These observations suggest that CK2 activity is required to maintain chondrocyte phenotype.

SIN-1 modulates TBB-induced cellular senescence in chondrocytes. Subsequently, the present study investigated the

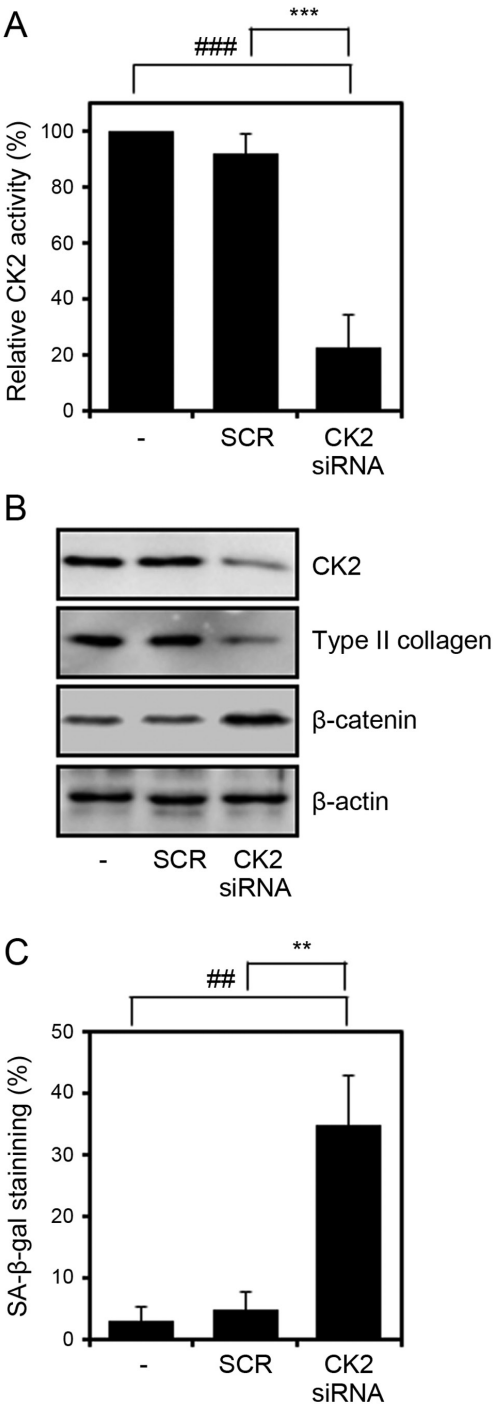

Figure 3. Effect of CK2 inhibition on chondrocyte senescence. Cells were transfected with CK2α siRNA, incubated for 48 h, and subjected to the (A) CK2 activity assay, (B) western blot analysis and (C) SA-β-gal assay. Values are presented as the mean ± standard deviation of three independent experiments. The P-value was calculated by one-way analysis of variance followed by Tukey's honest significant difference post hoc test. $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. negative control. **P<0.01 and ***P<0.001 vs. SCR. The difference between the negative control and SCR was not significant. siRNA, small interfering RNA; CK2, protein kinase casein kinase 2; TBB, 4,5,6,7-terabromo-2-azabenzimidazole; SA-β-gal, senescence-associated β-galactosidase; SCR, scrambled siRNA negative control.

effect of the peroxynitrite generator SIN-1, which is widely used to increase the expression and activity of HO-1 in several cell types, on TBB-induced chondrocyte senescence. In our previous study, it was reported that CK2 modulates

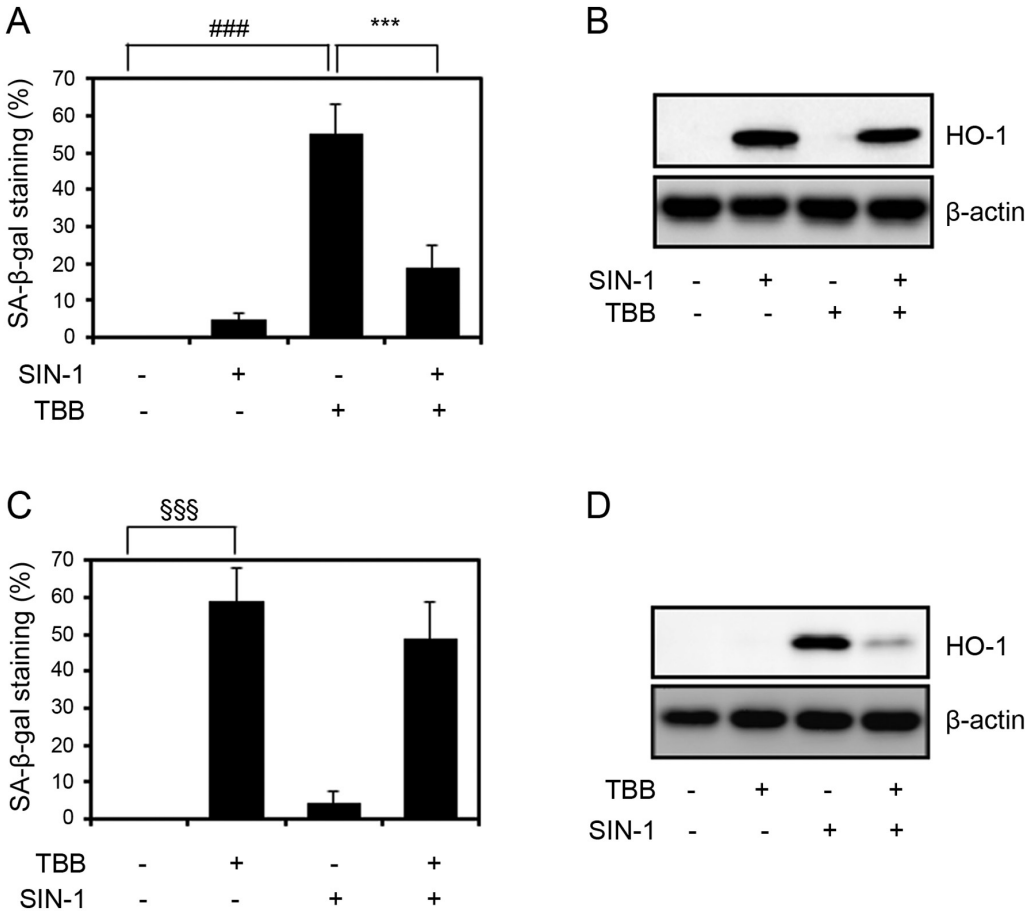

Figure 4. Effect of SIN-1 on TBB-induced chondrocyte senescence. (A) SA-β-gal assay and (B) western blot analysis of chondrocytes pretreated with 200 μM SIN-1 for 6 h, stimulated with 50 μM TBB, and incubated for 48 h. $^{###}$P<0.001: negative control vs. TBB; ***P<0.001: TBB vs. SIN-1 + TBB. P-values among the other groups were also analyzed (negative control vs. SIN-1: ns; negative control vs. SIN-1 + TBB: P<0.05; SIN-1 vs. TBB: P<0.001; SIN-1 vs. SIN-1 + TBB: P<0.05). (C) SA-β-gal assay and (D) western blot analysis of chondrocytes stimulated with 50 μM TBB for 6 h, and incubated for another 42 h in the absence or presence of SIN-1 (200 μM). §§§P<0.001: negative control vs. TBB. P-values among the other groups were also analyzed (negative control vs. SIN-1: ns; negative control vs. TBB + SIN-1: P<0.001; TBB vs. SIN-1: P<0.001; TBB vs. TBB + SIN-1: ns; SIN-1 vs. TBB + SIN-1: P<0.001). Values are presented as the mean ± standard deviation of three independent experiments. P-values were calculated by one-way analysis of variance followed by Tukey's honest significant difference post hoc test. For western blotting, β-actin was used as an internal control. SIN-1, 3-morpholinosydnonimine hydrochloride; TBB, 4,5,6,7-terabromo-2-azabenzimidazole; SA-β-gal, senescence-associated β-galactosidase; ns, not significant; HO-1, heme oxygenase-1.

SIN-1-induced HO-1 expression in chondrocytes (23), and as a result it was hypothesized that TBB-induced chondrocyte senescence was caused by the inhibition of HO-1. In the present study, the chondrocytes were cultured for 6 h either in medium alone or in medium containing 200 μM SIN-1 and stimulated with TBB (50 μM) for 48 h. As shown in Fig. 4A, pretreatment with SIN-1 suppressed TBB-induced cellular senescence, which resulted from the induction of the expression of HO-1 by pretreatment with SIN-1 (Fig. 4B). However, when the cells were treated with SIN-1 following TBB, no significant reduction in SA-β-gal staining was observed (Fig. 4C). In addition, as expected, pretreatment with TBB inhibited the induction of HO-1 by SIN-1 (Fig. 4D), indicating that the effect of SIN-1 on the TBB-induced senescence of chondrocytes is associated with the expression of HO-1.

HO-1 inhibits TBB-induced cellular senescence in chondrocytes. Based on the above-mentioned results, the enhancement of chondrocyte senescence by TBB treatment appeared to be associated with the expression of HO-1, therefore, an HO-1-overexpression vector was used to determine whether HO-1 directly modulates the senescence of TBB-treated chondrocytes. The chondrocytes were transfected with the HO-1 expression vector or control pcDNA vector and the protein overexpression of HO-1 was assessed by western blot analysis (Fig. 5A). As shown in Fig. 5B, the overexpression of HO-1 significantly reduced the senescence-specific SA-β-gal staining of TBB-treated chondrocytes. These results suggest that the forced overexpression of HO-1 decreased the CK2 inhibition-mediated senescence of chondrocytes.

Discussion

Under physiological conditions, chondrocytes have rare proliferative properties and have a central role in the make up of articular cartilage, which contains an avascular cartilage-specific matrix (25). Chondrocytes produce and maintain extracellular matrix, which is composed of type II collagen and sulfated proteoglycans, by facilitating the cell-matrix interactions responsible for several of the functions of cartilage (4,26). Although articular cartilage is hypoxic in nature, chondrocytes produce ROS to enable metabolism adapted to

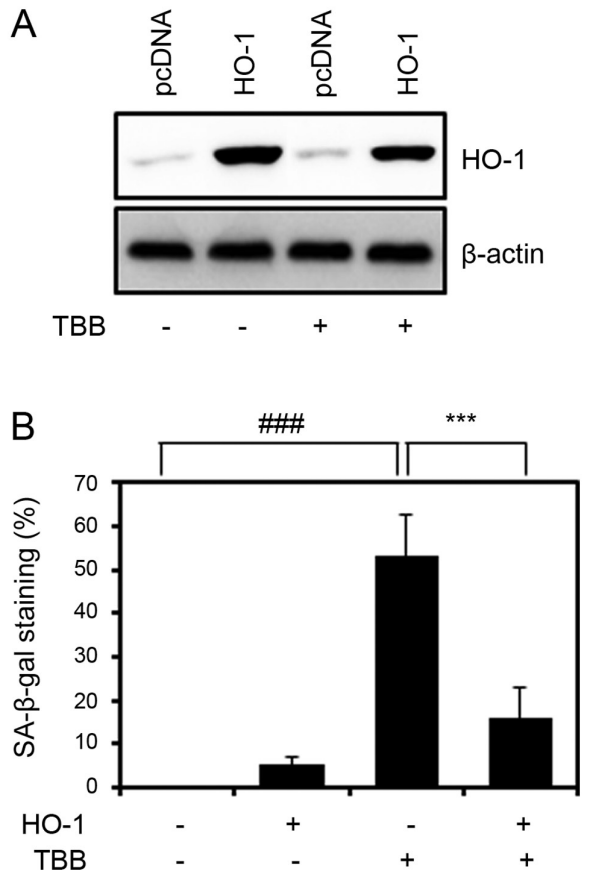

Figure 5. Effect of the overexpression of HO-1 on TBB-induced chondrocyte senescence. (A) Cells were subjected to western blot analysis to show the efficiency of HO-1 expression. β-actin was used as an internal control. (B) Cells were transfected with the HO-1 expression vector, incubated for 24 h, stimulated with 50 μM TBB for 48 h, and subjected to a SA-β-gal assay. Values are presented as the mean ± standard deviation of three independent experiments. P-values were calculated by one-way analysis of variance followed by Tukey's honest significant difference post hoc test. $^{###}$P<0.001: negative control vs. TBB, ***P<0.001: TBB vs. HO-1 + TBB. P-values among other groups are also analyzed (negative control vs. HO-1: ns; negative control vs. HO-1 + TBB: ns; HO-1 vs. TBB: P<0.001; HO-1 vs. HO-1 + TBB: ns). TBB, 4,5,6,7-terabromo-2-azabenzimidazole; SA-β-gal, senescence-associated β-galactosidase; ns, not significant; HO-1, heme oxygenase-1.

anaerobic conditions, and is exposed to abnormal levels of ROS produced by immune cells under pathological conditions, including OA, rheumatoid arthritis and gout (27-29). Chondrocytes constitutively express well-coordinated antioxidant enzymes, including superoxide dismutase (SOD; cytosolic Cu/Zn SOD and mitochondrial Mn SOD), glutathione peroxidase (GPX) and catalase (30,31).

Elevated levels of ROS and antioxidant depletion have been reported under pathological conditions, including those associated with inflammatory diseases (32). Oxidative stress can damage the components of the ECM by upregulating matrix metalloproteinases (MMPs) and inflammatory mediators, including inducible nitric oxide synthase and cyclooxygenase-2 (33,34), and by doing so can cause the apoptosis and senescence of chondrocytes in articular cartilage and destroy articular cartilage (9,13,35). Furthermore, age-related changes in the intracellular redox status of chondrocytes has been shown to be associated with the development of cell alterations (36,37). Apoptotic chondrocyte death is widely observed in degenerated cartilage of OA, and chondrocyte senescence may contribute to the reduction in chondrocyte numbers in articular cartilage; senescent chondrocytes accumulate with age and in articular cartilage of patients with OA (8,11-13).

Unlike SOD, GPX, and catalase, HO-1 is adaptively induced to protect chondrocytes and cartilage against the destructive effects of oxidative stress (10). It has also been reported that HO-1 inhibits catabolic enzymes and inflammatory cytokines that may contribute to the pathogeneses of cartilage diseases (5), which suggests that HO-1 is a component of defense systems that protect articular cartilage (27,38). In addition, it has been shown that HO-1 contributes significantly to protection against cartilage degeneration in humans (39). However, the mechanisms responsible for the regulation and activities of HO-1 in the contexts of chondrocyte maintenance, senescence and apoptosis in articular cartilage remain to be fully elucidated.

CK2 is critical in the control of cell proliferation, transformation and apoptosis (17-19), and reportedly, loss of CK2 activity is involved in chromatin structural alteration and changes in gene expression (40,41). Therefore, it appears that CK2 can stimulate the phosphorylation of several proteins required for DNA replication and transcription (15,42,43). In our previous study, it was reported that CK2 mediates the expression of HO-1 by phosphorylating and inducing the nuclear translocation of Nrf2 in chondrocytes exposed to oxidative stress (23). In the present study, it was found that the activity of CK2 is associated with the senescence of primary articular chondrocytes, and that the downregulation of CK2 induces cellular senescence by inhibiting the expression of HO-1. Furthermore, inhibition of CK2 activity, achieved using TBB (a specific CK2 inhibitor) or by silencing the CK2 gene, caused specific SA-β-gal staining of chondrocytes and altered the expression of type II collagen and β-catenin (phenotypic markers of chondrocyte differentiation). Subsequently, the present study investigated whether HO-1 is involved in TBB-induced chondrocyte senescence using the pharmacologic inducer (SIN-1) and by the transfection-induced overexpression of HO-1. Chondrocytes overexpressing HO-1 exhibited significant inhibition of TBB-induced cellular senescence, which provides evidence that the activity of HO-1 is associated with the CK2 inhibition-mediated senescence of chondrocytes, and suggests that HO-1 may act to inhibit chondrocyte senescence.

However, reports indicate that HO-1 has a dual role in pathological states, as high levels of HO-1 are frequently detected in pathological tissues (44,45). In parallel, chondrocytes obtained by serial subculture exhibit passage number-dependent increases in cellular senescence and expression of HO-1 (unpublished data), which may reflect age-related changes in intracellular redox status during the development of cellular senescence. This observation encourages the suggestion that the level of HO-1 in articular cartilage may be a useful biomarker of the degree of articular cartilage degeneration. Based on the results obtained in the present study, it can be concluded that the activity of CK2 may act as an anti-aging factor by inducing the expression of HO-1 to counteract the effects of chondrocyte senescence in articular cartilage.

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Availability of data and materials

All the datasets generated and analysed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

KMK, KK and YCP made contributions to the conception and design of the study. KMK, DHS and YCP performed the experiments. DHS, KK, and YCP performed the statistical analysis. DHS and YCP wrote and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments and protocols were approved by the Pusan National University Institutional Animal Care and Use Committee and performed in accordance with the institutional and national guidelines for the care and use of laboratory animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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