

Hyperglycemia increases the expression levels of sclerostin in a reactive oxygen species- and tumor necrosis factor- α -dependent manner

Jiho Kang¹, Kanitsak Boonantanasarn^{1,2}, Kyunghwa Baek³, Kyung Mi Woo¹, Hyun-Mo Ryoo¹, Jeong-Hwa Baek^{1,*}, Gwan-Shik Kim^{1,*}

¹Department of Molecular Genetics, School of Dentistry and Dental Research Institute, Seoul National University, Seoul, Korea

²Department of Anatomy, Faculty of Dentistry, Mahidol University, Bangkok, Thailand

³Department of Pharmacology, College of Dentistry, Gangneung-Wonju National University, Gangneung, Korea

Purpose: Sclerostin, an inhibitor of Wnt/ β -catenin signaling, exerts negative effects on bone formation and contributes to periodontitis-induced alveolar bone loss. Recent studies have demonstrated that serum sclerostin levels are increased in diabetic patients and that sclerostin expression in alveolar bone is enhanced in a diabetic periodontitis model. However, the molecular mechanism of how sclerostin expression is enhanced in diabetic patients remains elusive. Therefore, in this study, the effect of hyperglycemia on the expression of sclerostin in osteoblast lineage cells was examined.

Methods: C2C12 and MLO-Y4 cells were used in this study. In order to examine the effect of hyperglycemia, the glucose concentration in the culture medium was adjusted to a range of levels between 40 and 100 mM. Gene expression levels were examined by quantitative reverse transcription-polymerase chain reaction and Western blot assays. Top-Flash reporter was used to examine the transcriptional activity of the β -catenin/lymphoid enhanced factor/T-cell factor complex. Tumor necrosis factor- α (TNF α) protein levels were examined with the enzyme-linked immunosorbent assay. The effect of reactive oxygen species on sclerostin expression was examined by treating cells with 1 mM H₂O₂ or 20 mM N-acetylcysteine.

Results: The high glucose treatment increased the mRNA and protein levels of sclerostin. High glucose suppressed Wnt3a-induced Top-Flash reporter activity and the expression levels of osteoblast marker genes. High glucose increased reactive oxygen species production and TNF α expression levels. Treatment of cells with H₂O₂ also enhanced the expression levels of TNF α and sclerostin. In addition, N-acetylcysteine treatment or knockdown of TNF α attenuated high glucose-induced sclerostin expression.

Conclusions: These results suggest that hyperglycemia increases sclerostin expression via the enhanced production of reactive oxygen species and TNF α .

Keywords: Hyperglycemia, Osteoblasts, Reactive oxygen species, Tumor necrosis factor- α .

INTRODUCTION

Diabetes mellitus (DM) is among the primary risk factors for periodontitis [1]. DM potentiates the severity of periodontitis and further accelerates alveolar bone destruction. Type 2 DM is the most common form of diabetes, characterized by hyperglycemia, insulin resistance, and relative insulin deficiency. Recent studies have demonstrated that bone loss and osteoporotic fracture risk are increased in DM patients [2]. Several mechanisms have been suggested to explain the increased bone loss and fracture risk in DM patients. Abnormal cal-

pISSN 2093-2278
eISSN 2093-2286



JPIS >
Journal of Periodontal
& Implant Science

Research Article

J Periodontal Implant Sci 2015;45:101-110
<http://dx.doi.org/10.5051/jpis.2015.45.3.101>

Received: Apr. 30, 2015

Accepted: May 19, 2015

*Correspondence:

Gwan-Shik Kim

Department of Molecular Genetics, Seoul National University School of Dentistry, 101 Daehak-ro, Jongno-gu, Seoul 110-744, Korea

E-mail: gskim@snu.ac.kr

Tel: +82-2-740-8687

Fax: +82-2-741-3193

*Co-correspondence: Jeong-Hwa Baek

Department of Molecular Genetics, Seoul National University School of Dentistry, 101 Daehak-ro, Jongno-gu, Seoul 110-744, Korea.

E-mail: baekjh@snu.ac.kr

Tel: +82-2-740-8688

Fax: +82-2-741-3193

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0/>).

cium metabolism occurs in patients with DM. Increased urinary excretion of calcium and decreased intestinal calcium absorption can cause a negative calcium balance with secondary hyperparathyroidism and increased bone remodeling, contributing to a lower bone mineral density in DM patients [3]. Diabetic animals produce sufficient amounts of immature mesenchymal tissue but fail to adequately express Runx2 and Dlx5, the transcription factors for osteoblast differentiation [4]. Advanced glycation end products (AGEs) also have been implicated in the development of DM-related osteopenia. In DM patients, a larger amount of AGEs is accumulated in bone tissue. AGEs inhibit the synthesis of bone matrix proteins such as type I collagen and osteocalcin, and decrease mineralized nodule formation in osteoblasts [5-7]. AGEs have also been suggested as a biomarker for the increased risk of fractures because AGEs have been shown to be associated with bone rigidity [8]. The receptor for AGEs is expressed in bone cells and its activation stimulates the production of reactive oxygen species (ROS), activating the NF- κ B signaling pathway [9]. Intracellular ROS-mediated oxidative stress plays a crucial role in bone health. Increased ROS-mediated oxidative stress in osteoblasts and osteocytes contributes to the cell death of osteocytes and mechanical unloading-induced bone loss [10,11]. Patients with type 2 DM have been found to show elevated levels of mitochondrial ROS, which contribute to elevated osteolysis and bone fractures in DM patients [12]. Multiple factors, including a negative calcium balance, microvascular damage, AGEs, and ROS, may contribute to the increased bone loss and fracture risk of DM patients, but further studies are necessary to clarify the mechanisms involved in DM-associated osteopenia and fracture risk.

The canonical Wnt signaling pathway controls cell proliferation and differentiation by regulating the degradation and localization of β -catenin. The binding of canonical Wnt ligands to Frizzled and low-density lipoprotein receptor-related protein (LRP5/6) inactivates β -catenin degradation complexes, resulting in the accumulation and nuclear translocation of β -catenin. In the nucleus, β -catenin induces the transcriptional activation of target genes by forming a complex with lymphoid-enhanced factor/T-cell factor (LEF/TCF). Canonical Wnt signaling promotes the differentiation of mesenchymal progenitor cells into osteoblasts. In osteoblasts, the Wnt pathway induces cell proliferation, differentiation, and matrix mineralization, playing a pivotal role in bone development and formation [13]. Sclerostin, a protein encoded by the *Sost* gene, acts as an antagonist to Wnt signaling by binding to LRP5/6 co-receptors and inhibiting the binding of Wnt ligands [14]. Sclerostin is mainly produced by osteocytes [15]. Sclerostin-mediated inhibition of Wnt signaling affects osteoblast differentiation and bone formation [16]. Recent reports have demonstrated that removing or blocking the function of sclerostin ameliorated alveolar bone defects in an experimentally induced periodontitis model, suggesting that sclerostin plays an important role in periodontitis-associated bone destruction [17-19].

We have previously demonstrated that serum sclerostin levels were higher in mice fed with a high-fat diet than control mice, and that tumor necrosis factor α (TNF α) mediates the induction of

sclerostin expression in mice fed with a high-fat diet [20]. High fat diet-fed mice were shown to exhibit higher serum glucose levels and glucose intolerance compared to their healthy littermates. Similarly, serum sclerostin levels have been found to be significantly higher in both type 1 and type 2 DM patients independently of gender and age, compared to non-diabetic controls [21-23]. These reports suggest that increased sclerostin expression contributes to DM-associated osteopenia and accelerated alveolar bone loss in diabetic periodontitis patients. However, the molecular mechanisms involved in the DM-mediated induction of sclerostin expression remain elusive. In this study, we investigated how hyperglycemia regulates the expression of sclerostin in osteoblasts and osteocytes using cells from the murine cell lines C2C12 and MLO-Y4.

MATERIALS AND METHODS

Materials

The easy-BLUE™ and StarTaq™ reagents were ordered from iNTRON Biotechnology (Seongnam, Korea) and the AccuPower RT-Pre Mix was purchased from Bioneer (Daejeon, Korea). The SYBR Premix ExTaq™ was purchased from TaKaRa (Otsu, Japan). Tissue culture medium and serum were purchased from HyClone (Logan, UT, USA) and BioWhittaker (Walkersville, MD, USA). A Bright-Glo luciferase assay kit was obtained from Promega (Madison, WI, USA). Recombinant bone morphogenetic protein 2 (BMP2) and Wnt3a were purchased from R&D Systems (Minneapolis, MN, USA) and added to the culture medium at a concentration of 50 ng/mL. *D*-(+)-Glucose and H₂O₂ were ordered from Sigma (St. Louis, MO, USA).

Cell culture

C2C12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μ g/mL streptomycin. The cell culture medium contained 5 mM glucose. In order to induce osteoblastic differentiation, the C2C12 cells were incubated in DMEM supplemented with 50 ng/mL BMP2, 5% FBS, 100 U/mL penicillin, and 100 μ g/mL streptomycin after two days of confluence. Osteocytic MLO-Y4 cells were cultured in alpha modified Eagle's medium (α MEM) supplemented with 5% calf serum and 5% FBS.

Reverse transcription-polymerase chain reaction

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed to examine mRNA expression levels. Total RNA was isolated by using an easyBLUE reagent and cDNA was synthesized from the total RNA using the AccuPower RT PreMix, and subsequently used for quantitative real time-PCR amplification using SYBR Premix EX Taq. Each sample was analyzed in triplicate, and the target genes were normalized to the reference housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR primer sequences used for real-time PCR were as follows: *Sost*, (f) 5'-AGCCTTCAGGAATGATGCCAC-3' and (r) 5'-CTTTGGCGT-CATAGGGATGGT-3'; TNF α , (f) 5'-GACGACAGCAAGGGACTAGC-3'

and (r) 5'-GCTTTCTGTGCTCATGGTGC-3'; Runx2, (f) 5'-TTCTCAACCCACGAATGCAC-3' and (r) 5'-CAGGTACGTGGTAGTGAGT-3'; osterix, (f) 5'-CCCACCTTCCCTCACTC-3' and (r) 5'-CCTGTACCACGAGCCAT-3'; alkaline phosphatase, (f) 5'-CCAACCTTTTGTGCCAG-3' and (r) 5'-GGCTACAATTGGTGTGAGCTTTT-3'; osteocalcin, (f) 5'-CTGACAAAGCCTTCATGT-3' and (r) 5'-GCGCCGGAGTCTGTTCAC-3'; and GAPDH, (f) 5'-TCAATGACAACCTTGTCAAGC-3' and (r) 5'-CCAGGGTTTCTACTCCTTGG-3'.

Western blot analysis

In order to prepare the whole-cell lysate, cells were lysed in the PRO-PREP (iNtRON Biotechnology) protein extraction solution, which contains protease inhibitors, including PMSF (174 µg/mL), pepstatin A (0.7 µg/mL), leupeptin (2.0 µg/mL), and aprotinin (0.5 µg/mL). The lysates were then sonicated briefly and centrifuged at 16,000 × *g* for 10 minutes, and the supernatants were used for Western blot analysis. Each sample containing equal amounts of protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins separated in the gel were subsequently transferred onto a polyvinylidene fluoride membrane. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween20, incubated with sclerostin or actin antibodies, and subsequently incubated with horseradish peroxidase-conjugated secondary antibody. Immune complexes were visualized using the Suprex reagent and luminescence was detected with a LAS1000 machine (Fuji PhotoFilm; Tokyo, Japan).

Enzyme-linked immunosorbent assay

Cells were incubated for 48 hours in the medium supplemented with high glucose or H₂O₂. Whole cell lysates were prepared as described above and the expression levels of TNFα protein in the cell lysates were determined using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Koma Biotech, Seoul, Korea) according to the manufacturer's instructions.

Knockdown of TNFα

siGENOME ON-TARGETplus SMARTpool mouse TNFα siRNA and non-targeting control scrambled siRNA were purchased from Dharmacon (Lafayette, CO, USA). Cells were transfected with siRNA using Dharmafect (Dharmacon, Lafayette, CO, USA) according to the manufacturer's instructions. The efficacy of the knockdown was assessed by quantitative RT-PCR.

Luciferase reporter assays

The transcriptional activity of β-catenin was examined using a Top-Flash luciferase reporter. MLO-Y4 cells were plated into 96-well plates at a density of 2 × 10⁴ cells/well. The cells were transiently transfected with 0.2 µg of Top-Flash plasmid using the Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA). Cells were then incubated for 72 hours in the presence or absence of high glucose or Wnt3a (50 ng/mL). Luciferase activity was measured using the Bright-Glo luciferase assay kit according to the manufacturer's instructions.

Dichlorofluorescein diacetate assay for measuring intracellular ROS production

Assays were performed as described previously [24], with some modifications. The cells were grown to 100% confluence and incubated in the presence of 2 µM dichlorofluorescein diacetate (DCF-DA) in the dark at room temperature for 10 minutes. The emitted fluorescence was then measured at 490 nm, and this value was used as a baseline for the following experiments. The cells were then incubated in the presence or absence of high glucose, TNFα, or H₂O₂ for the indicated periods.

Statistical analysis

The data were presented as the mean ± standard deviation. The statistical significance of the results was assessed by one-way or two-way analysis of variation using Prism6 (GraphPad Software Inc., La Jolla, CA, USA). Post-hoc analysis was performed using the Sidak or Tukey's multiple comparisons test to explore the differences among individual means. *P*-values < 0.05 were considered to indicate statistical significance.

RESULTS

High glucose increases sclerostin expression

C2C12 is a mouse myoblast cell line that can be differentiated into osteoblasts by treatment with BMP2 [25]. In order to examine the effect of high glucose on the expression levels of sclerostin, osteoblastic differentiation of C2C12 cells was induced by incubating confluent cells in the presence of 50 ng/mL BMP2. With the initiation of osteogenic differentiation, the C2C12 cells were exposed to varying concentrations of high glucose (40–100 mM) for 48 hours. Quantitative RT-PCR results demonstrated that high glucose increased mRNA levels of sclerostin at all of the concentrations examined, but statistical significance was observed at glucose concentrations higher than 40 mM (Fig. 1A). The fold changes of sclerostin mRNA in the 5, 40, 60, 80, and 100 mM glucose samples were 1.45 ± 0.26, 2.14 ± 0.15, 7.03 ± 1.24, 6.90 ± 0.13, and 15.85 ± 1.71, respectively. However, Western blot analysis showed that the increase in sclerostin protein levels was most significant at the 100 mM concentration (Fig. 1B). Therefore, subsequent experiments were performed using a glucose concentration of 100 mM. High glucose (100 mM)-induced sclerostin expression was also observed in the C2C12 cells after incubation for 24 hours (Fig. 1C).

Since sclerostin is mainly expressed in osteocytes, the effect of high glucose on sclerostin expression was also examined using the MYO-Y4 osteocytic cell line. Similar to the results obtained from the C2C12 cells, 100 mM glucose significantly increased sclerostin expression as assessed by both mRNA and protein levels (Fig. 1D, E). The fold changes of sclerostin mRNA in the 5, 40, and 100 mM glucose samples were 1.00 ± 0.05, 1.38 ± 0.18 and 5.82 ± 0.37, respectively. Interestingly, in MLO-Y4 cells, 40 mM glucose clearly increased sclerostin expression as reflected in the protein levels, but not in the mRNA levels.

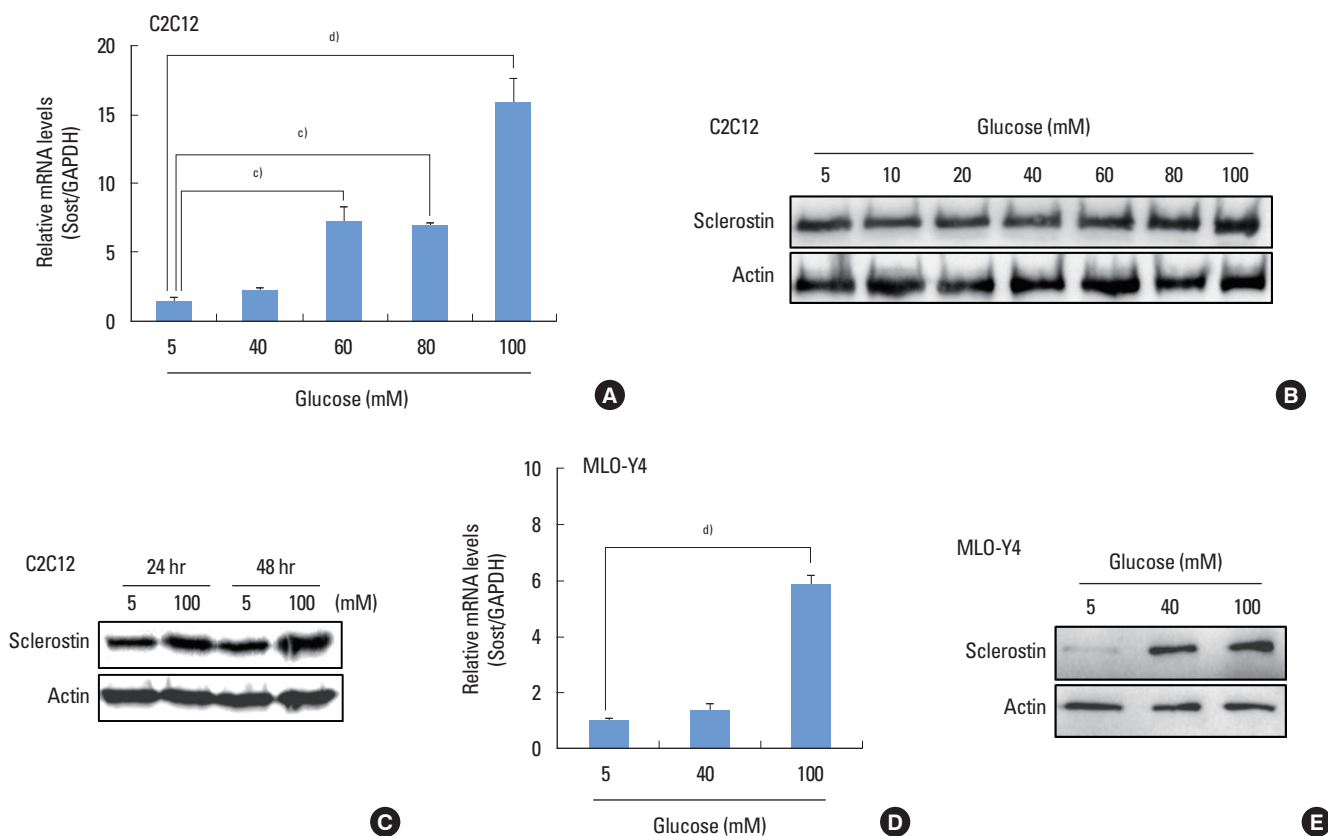


Figure 1. High glucose increases sclerostin expression. (A–C) C2C12 cells were cultured in osteogenic medium in the presence or absence of high glucose for 48 hours (h) unless specified, and (A) RT-PCR and (B) Western blot analysis were performed. (D, E) MLO-Y4 cells were incubated for 48 hours in the presence or absence of high glucose treatment, followed by (D) quantitative RT-PCR and (E) Western blot analysis. The graphs (A, D) indicate the mean \pm standard deviation of the triplicate samples (^c $P < 0.001$, ^d $P < 0.0001$). GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction. In the Figure 1C, 5 and 100 mM indicate the concentration of glucose in the culture medium.

High glucose inhibits Wnt/ β -catenin signaling

Since high glucose increased the expression levels of sclerostin, we next examined whether high glucose suppresses Wnt/ β -catenin signaling. C2C12 cells were incubated for 24 hours in the presence and absence of Wnt3a (50 ng/mL) or high glucose (100 mM), and the expression levels of osteogenic marker genes, including ALP, Runx2, osteocalcin, and osterix, were examined. Wnt3a significantly enhanced the expression levels of all the marker genes examined (Fig. 2A). In addition, high glucose significantly attenuated marker gene expression levels both in basal and in Wnt3a-treated cells (Fig. 2A). The Wnt3a-induced gene expression levels in 5 mM glucose compared to 100 mM glucose were as follows: Runx2, 7.76 ± 1.08 vs. 4.55 ± 0.14 ; osterix, 29.65 ± 3.87 vs. 16.57 ± 0.82 ; ALP 21.82 ± 1.76 vs. 9.05 ± 0.14 ; and osteocalcin, 5.93 ± 0.19 vs. 3.06 ± 0.08 .

Since MLO-Y4 cells are osteocytic cells, we directly examined the effect of high glucose on Wnt/ β -catenin signaling using the Top-Flash luciferase assay in MLO-Y4 cells, rather than examining the effects on osteogenic marker genes. Since 40 mM glucose increased the sclerostin protein levels in MLO-Y4 cells, we used this concentration to further check the functional effect of high glucose on

Wnt/ β -catenin signaling. Wnt3a significantly increased Top-Flash activity, which was blocked by treatment for three days with 40 mM glucose (Fig. 2B). The Wnt3a-induced Top-Flash activities in 5 mM glucose compared to 100 mM glucose were 308.50 ± 65.88 vs. 141.50 ± 19.49 .

High glucose-induced ROS production contributes to enhanced sclerostin expression

We next examined whether high glucose enhances the production of ROS. Intracellular ROS levels were determined using DCF-DA. H_2O_2 (1 mM) was used as a positive control. Compared to the control cells, the ROS production was significantly higher in high glucose- and H_2O_2 -treated cells (Fig. 3). The ROS levels in the control, 100 mM glucose, and 1 mM H_2O_2 groups after two hours of incubation were $141,756 \pm 19,110$, $257,520 \pm 14,679$, and $443,844 \pm 16,675$, respectively. In order to explore whether increased ROS levels contribute to sclerostin expression, C2C12 cells were incubated with 0.1–1 mM H_2O_2 for 48 hours and the expression levels of sclerostin were determined. Quantitative RT-PCR results demonstrated that H_2O_2 significantly increased sclerostin mRNA levels in a dose-dependen-

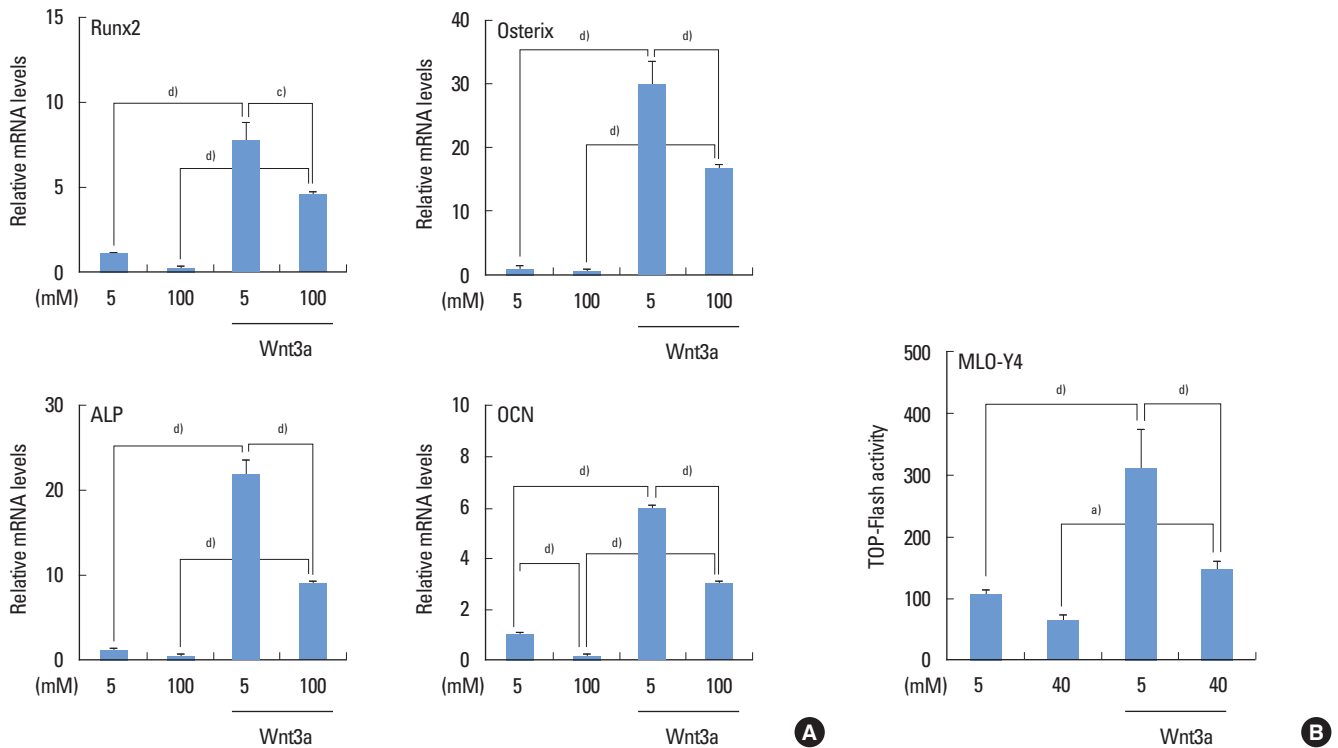


Figure 2. High glucose negatively regulates Wnt/ β -catenin signaling. (A) C2C12 cells were incubated for 24 hours in the presence or absence of Wnt3a (50 ng/ml) or high glucose (100 mM), followed by quantitative reverse transcription-polymerase chain reaction of osteogenic marker genes. (B) MLO-Y4 cells were transiently transfected with the β -catenin expression vector and Top-Flash luciferase reporter and cultured in the presence or absence of Wnt3a or high glucose (40 mM) for 72 hours. The graphs indicate the mean \pm standard deviation of (A) the triplicate or (B) quadruplicate samples (^a $P < 0.05$, ^c $P < 0.001$, ^d $P < 0.0001$). In the figures, 5 and 100 mM indicate the concentration of glucose in the culture medium.

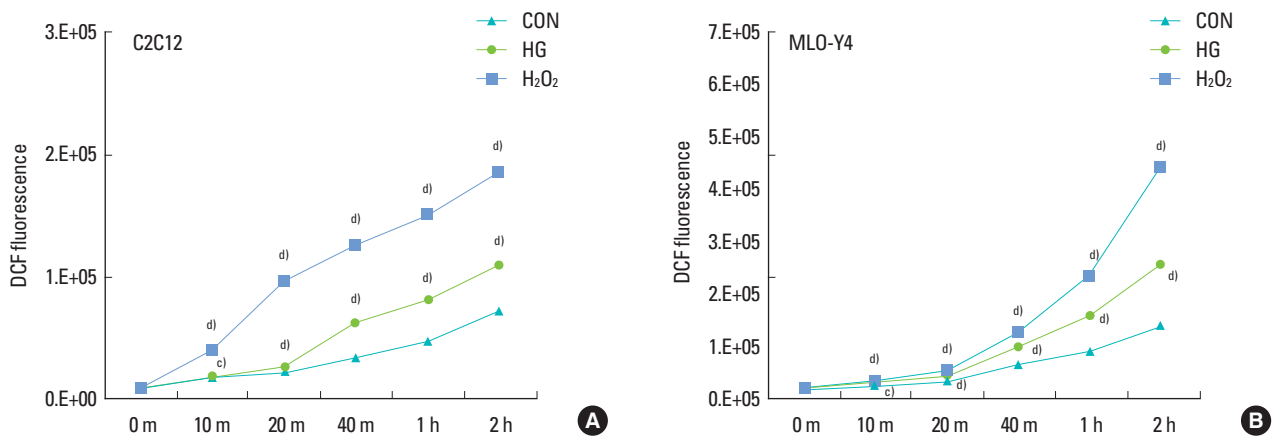


Figure 3. High glucose enhances reactive oxygen species production. Dichlorofluorescein diacetate was added to the culture medium, and (A) C2C12 and (B) MLO-Y4 cells were treated with high glucose (100 mM) or H₂O₂ (1 mM) for the indicated time periods, followed by measuring fluorescence from oxidized dichlorofluorescein. The graphs indicate the mean \pm standard deviation of the octuplicate samples (^c $P < 0.001$, ^d $P < 0.0001$; significantly different from the control group at each time point). DCF, dichlorofluorescein; CON, control; HG, high glucose; m, minutes; h, hours.

dent manner (Fig. 4A). The fold changes of sclerostin mRNA in 0, 0.1, 0.3, and 1.0 mM H₂O₂ samples were 0.87 ± 0.05 , 1.50 ± 0.16 , 2.26 ± 0.29 , and 3.17 ± 0.30 , respectively. In order to further clarify the role of ROS production in high glucose-induced sclerostin expression, C2C12 and MLO-Y4 cells were cultured in the presence or absence

of an antioxidant, N-acetylcysteine (20 mM). The addition of N-acetylcysteine significantly suppressed high glucose- and H₂O₂-induced sclerostin expression as assessed by both mRNA and protein levels (Fig. 4B-E). High glucose-induced sclerostin mRNA levels in the vehicle compared to N-acetylcysteine groups were 3.01 ± 0.20 vs.

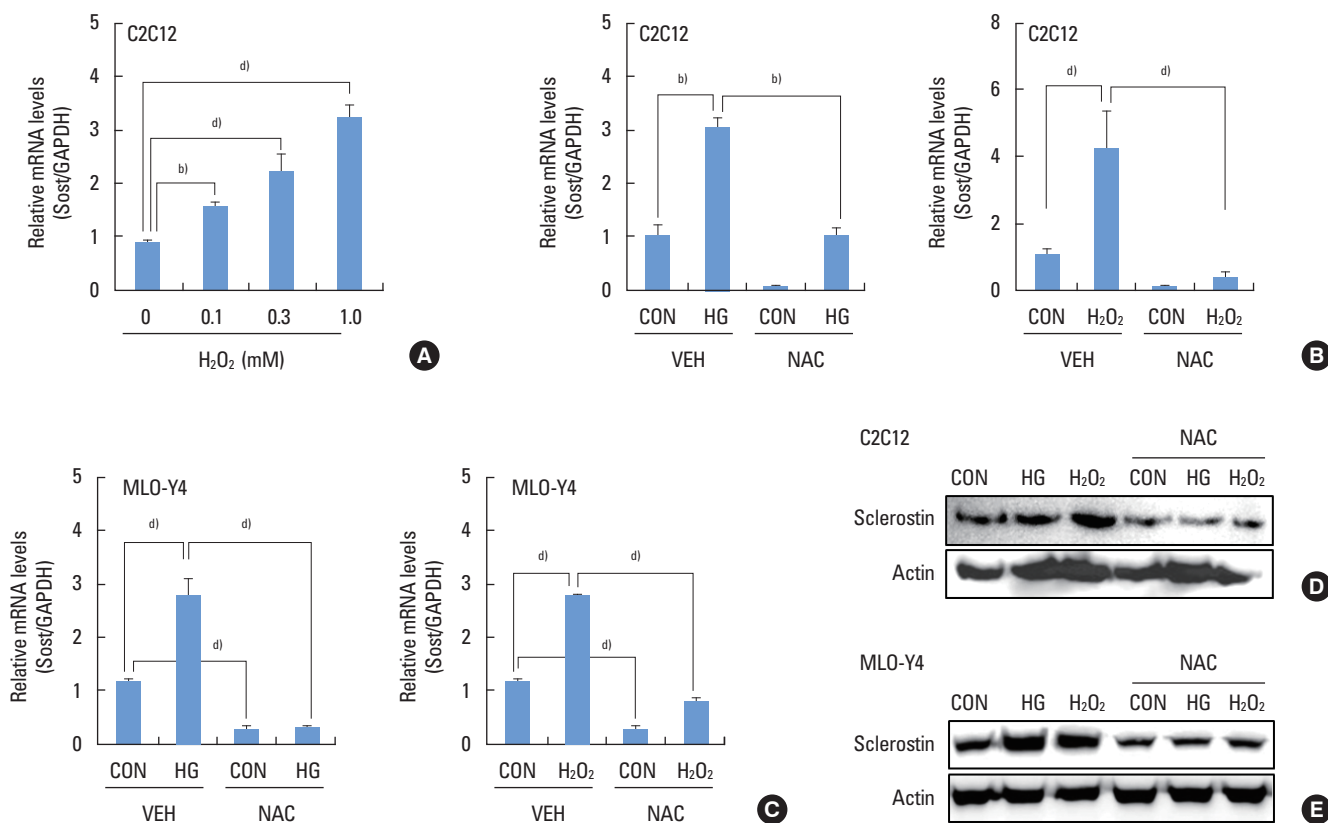


Figure 4. Reactive oxygen species contribute to high glucose-induced sclerostin expression. (A) C2C12 cells were incubated for 48 hours in the presence of H₂O₂ at the indicated concentrations, followed by (B–E) quantitative RT-PCR for sclerostin. (B, D) C2C12 and (C, E) MLO-Y4 (C, E) cells were incubated for 48 hours in the presence or absence of the indicated reagents, followed by (B, C) quantitative RT-PCR and (D, E) Western blot analysis (D, E). The graphs (A–C) indicate the mean ± standard deviation of the three to five samples (^b*P*<0.01, ^d*P*<0.0001). CON (control), 5 mM glucose; HG (high glucose), 100 mM glucose; H₂O₂, 1 mM H₂O₂; NAC, 20 mM N-acetylcysteine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction.

1.05 ± 0.10 in C2C12 cells and 2.74 ± 0.35 vs. 0.27 ± 0.04 in MLO-Y4 cells. H₂O₂-induced sclerostin mRNA levels in the vehicle compared to the N-acetylcysteine groups were 4.18 ± 1.19 vs. 0.41 ± 0.11 in the C2C12 cells and 2.70 ± 0.10 compared to 0.79 ± 0.04 in the MLO-Y4 cells. These results suggest that high glucose-induced ROS production contributes to the enhanced expression of sclerostin.

TNF α contributes to high glucose-induced sclerostin expression

Since a previous report has demonstrated that TNF α enhances sclerostin expression in MLO-Y4 cells [20], we examined whether high glucose stimulates TNF α expression. Both high glucose and H₂O₂ significantly increased the expression levels of the TNF α protein (Fig. 5A, B). Since H₂O₂ also exerted a stimulatory effect on TNF α expression, we examined whether antioxidant treatment attenuates high glucose-induced TNF α expression. The addition of N-acetylcysteine significantly downregulated high glucose- and H₂O₂-induced TNF α expression (Fig. 5C, D). High glucose-induced TNF α mRNA levels in the vehicle compared to N-acetylcysteine groups were 18.61 ± 0.62 vs. 7.13 ± 0.68 in the C2C12 cells and

3.25 ± 0.12 vs. 0.32 ± 0.02 in the MLO-Y4 cells. H₂O₂-induced TNF α mRNA levels in the vehicle compared to N-acetylcysteine groups were 7.74 ± 0.93 vs. 2.54 ± 0.06 in the C2C12 cells and 2.67 ± 0.05 vs. 0.29 ± 0.02 in the MLO-Y4 cells. These results suggest that ROS are involved in high glucose-induced TNF α expression.

In order to further examine whether TNF α mediates high glucose-induced sclerostin expression, the knockdown of TNF α was induced using TNF α siRNA. Quantitative RT-PCR results showed that TNF α siRNA suppressed high glucose- and H₂O₂-induced TNF α expression (Fig. 6A–D). Compared to control siRNA-transfected cells, high glucose and H₂O₂-induced sclerostin expression was significantly attenuated in TNF α siRNA-transfected cells (Fig. 6A–D). High glucose-induced sclerostin mRNA levels in the control siRNA compared to TNF α siRNA groups were 2.38 ± 0.35 vs. 1.10 ± 0.41 in the C2C12 cells and 2.90 ± 0.13 vs. 0.64 ± 0.21 in the MLO-Y4 cells. The H₂O₂-induced sclerostin mRNA levels in the control siRNA compared to TNF α siRNA groups were 9.39 ± 0.92 vs. 1.03 ± 0.07 in the C2C12 cells and 2.75 ± 0.59 vs. 1.46 ± 0.37 in the MLO-Y4 cells. These results suggest that high glucose-induced ROS increase TNF α expression, which subsequently enhances sclerostin expression.

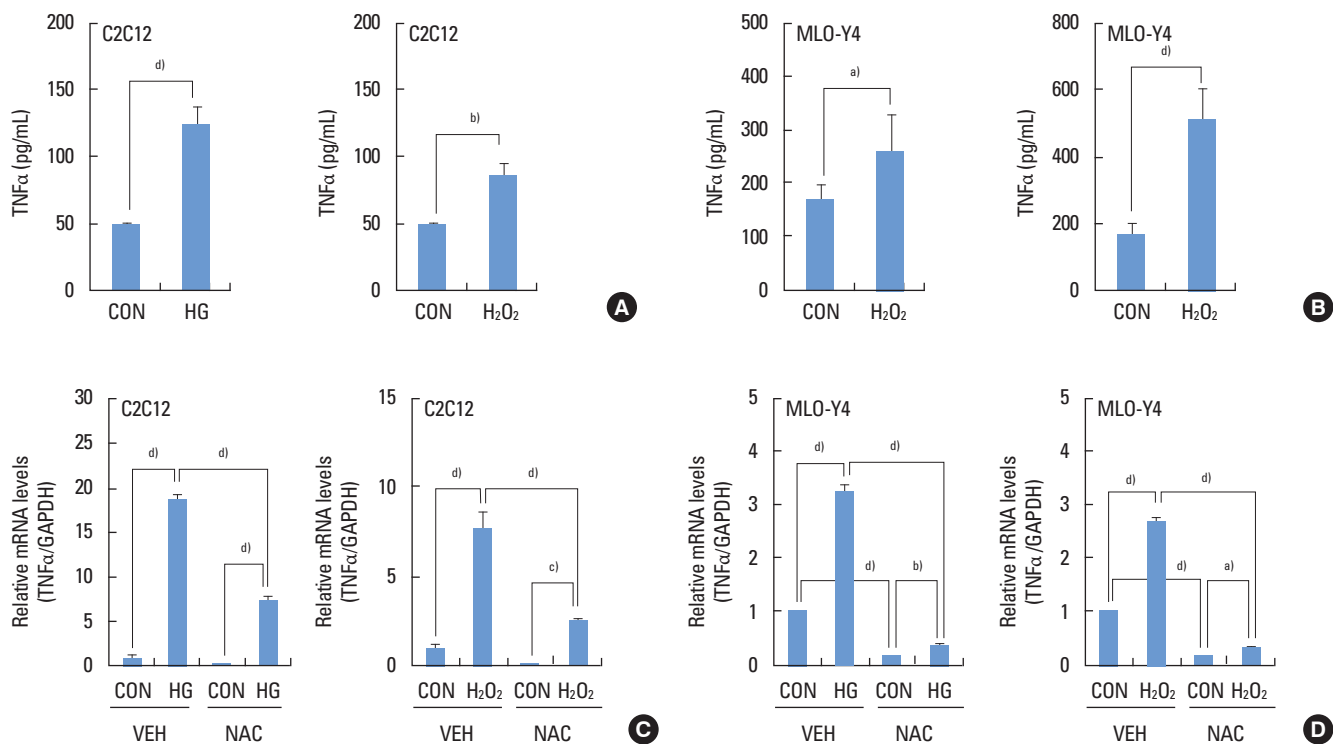


Figure 5. High glucose and reactive oxygen species increase TNF α expression. (A, C) C2C12 and (B, D) MLO-Y4 cells were incubated for 48 hours in the presence of the indicated reagents. (A, B) An ELISA assay for TNF α was then performed using cell lysates, and (C, D) RT-PCR for TNF α was performed. The graphs indicate the mean \pm standard deviation of the three to eight samples (^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$, ^d $P < 0.0001$). TNF α , tumor necrosis factor- α ; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcription-polymerase chain reaction; CON, control; HG, high glucose; VEH, vehicle; NAC, N-acetylcysteine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

DISCUSSION

Sclerostin is an inhibitor of Wnt/ β -catenin signaling and exerts negative effects on bone formation. Hyperglycemia is a representative symptom of DM. Recent studies demonstrated that the risk for osteoporotic fracture and serum sclerostin levels are increased in DM patients and that osteocytic expression of sclerostin is increased in diabetic rats with periodontitis [2,26]. However, the molecular mechanism of how sclerostin expression is enhanced in diabetic patients remains elusive. In this study, we demonstrated that the exposure of osteoblast lineage cells to high glucose induces ROS and TNF α production, which subsequently upregulates the expression levels of sclerostin.

In this study, we examined the effect of high glucose by adding extra glucose to the culture medium and adjusting the glucose concentration up to 100 mM. In order to rule out the possibility that the high glucose-induced effect on sclerostin resulted from high osmolarity, we compared the effect of high glucose (100 mM) with that of high mannitol (100 mM). Compared to high glucose, the regulatory effect of mannitol on sclerostin and TNF α expression was insignificant, suggesting that the regulatory effect of high glucose on sclerostin and TNF α expression is independent of osmolarity (data not shown). The stimulatory effect of high glucose on scleros-

tin expression in this study is consistent with the results from a recently published report [27]. In this report, Tanaka et al. [27] demonstrated that treating MLO-Y4-A2 cells with 22 mM glucose for 48 hours significantly increased sclerostin expression, as measured by both mRNA and protein levels.

Chronic hyperglycemia can directly induce an inflammatory state, in which increased levels of cytokines can lead to the degradation of the pancreatic beta cells in both type 1 and type 2 DM [28]. In DM patients, the serum levels of inflammatory cytokines, including IL-6, IL-18, IL-1, and TNF α , are significantly increased [29]. Similar to this *in vivo* phenomenon, the exposure of C2C12 and MLO-Y4 cells to high glucose significantly increased the expression levels of TNF α mRNA and protein. The induction of TNF α expression by high glucose was blocked by N-acetylcysteine, suggesting that ROS production is necessary for high glucose-induced TNF α expression. TNF α plays an important role in inflammatory bone loss, including rheumatoid arthritis, periodontitis, and osteoporosis [30,31]. TNF α also inhibits osteogenic differentiation and bone formation [32,33]. Since TNF α also enhances sclerostin expression in a NF- κ B-dependent manner in osteocytes [20], it is suggested that enhanced TNF α production by high glucose contributes to increased alveolar bone loss in DM patients.

In DM patients, a greater amount of AGEs is accumulated. The

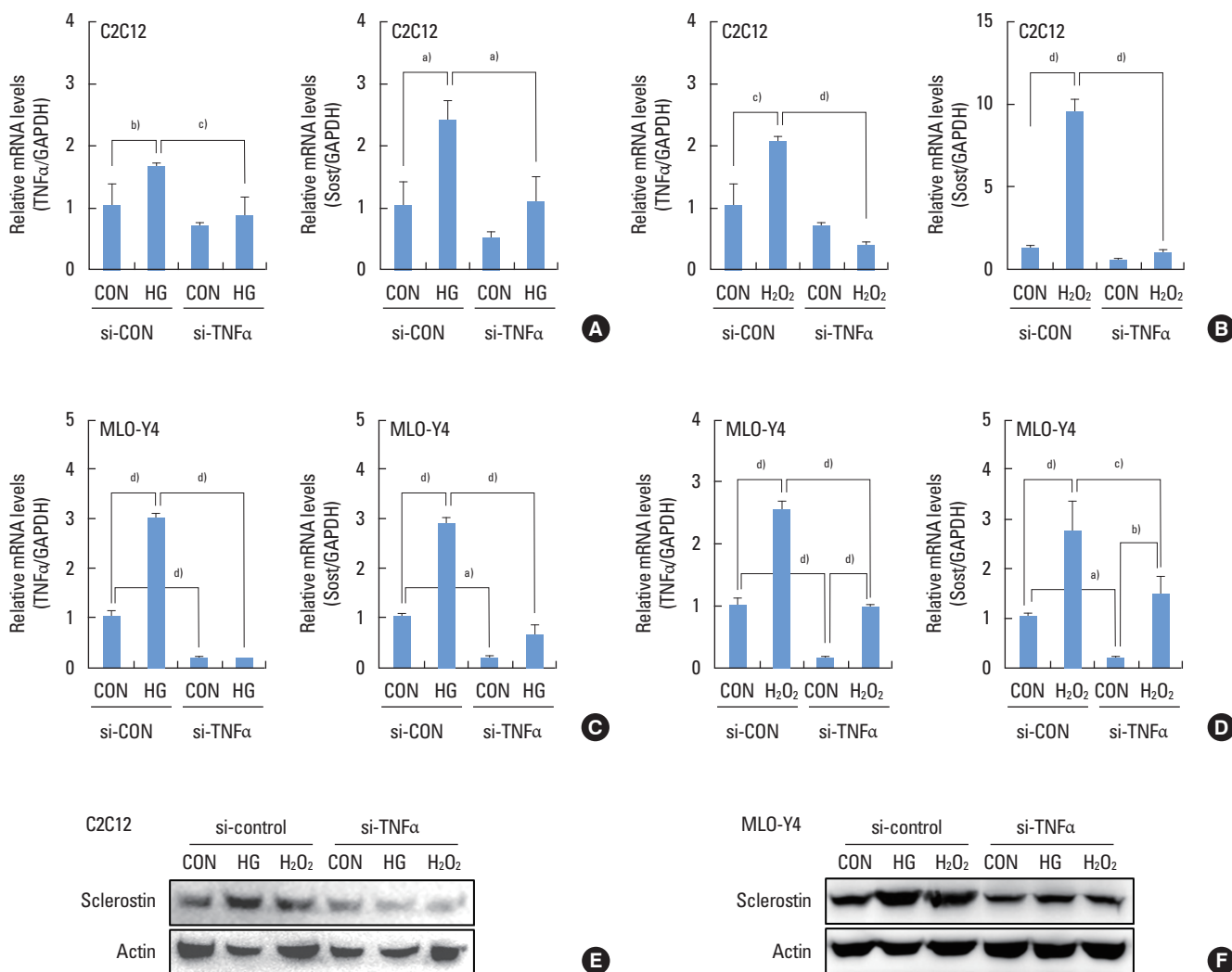


Figure 6. TNF α mediates high glucose-induced sclerostin expression. (A, B, E) C2C12 and (C, D, F) MLO-Y4 cells were transiently transfected with control siRNA or TNF α siRNA, followed by incubation for 48 hours in the presence or absence of the indicated reagents. (A–D) Quantitative RT-PCR for TNF α and sclerostin, and (E, F) Western blot analysis were then performed. The graphs indicate the mean \pm standard deviation of the triplicate samples (^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$, ^d $P < 0.0001$). TNF α , tumor necrosis factor- α ; RT-PCR, reverse transcription-polymerase chain reaction; CON, control; HG, high glucose; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

activation of the RAGE receptor by AGEs stimulates the production of ROS and activates the NF- κ B signaling pathway [9]. In the present study, high glucose increased ROS production in both C2C12 and MLO-Y4 cells, and the addition of an antioxidant prevented high glucose-induced TNF α and sclerostin expression. In addition, Tanaka et al. [27] demonstrated that AGEs enhance the expression levels of sclerostin in MLO-Y4-A2 cells. Therefore, it is suggested that both AGEs and hyperglycemia contribute to the increase in serum sclerostin levels in DM patients by increasing ROS production.

In conclusion, in this study, it was demonstrated that exposing osteoblast lineage cells to high glucose enhanced ROS and TNF α production, which subsequently induced sclerostin expression. Considering that TNF α and oxidative stress are among the main

mediators that enhance osteoclastic bone resorption but suppress osteoblastic bone formation in many inflammatory diseases, the data from in this study further support the hypothesis that the regulation of TNF α and oxidative stress levels in diabetic patients may be of therapeutic relevance for alveolar bone complications derived from chronic hyperglycemia.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

ACKNOWLEDGEMENTS

This study was supported by the Basic Science Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Science, ICT & Future Planning (NRF-2012R1A1A2003385), and by a NRF grant through the Oromaxillofacial Dysfunction Research Center for the Elderly (No. 2013070465) at Seoul National University in Korea.

ORCID

Jiho Kang <http://orcid.org/0000-0001-7789-9251>
 Kanitsak Boonantananasarn <http://orcid.org/0000-0001-9288-0786>
 Kyunghwa Baek <http://orcid.org/0000-0002-6290-0392>
 Kyung Mi Woo <http://orcid.org/0000-0002-2782-1189>
 Hyun-Mo Ryoo <http://orcid.org/0000-0001-6769-8341>
 Jeong-Hwa Baek <http://orcid.org/0000-0002-2032-1683>
 Gwan-Shik Kim <http://orcid.org/0000-0002-1175-572X>

REFERENCES

1. Wu YY, Xiao E, Graves DT. Diabetes mellitus related bone metabolism and periodontal disease. *Int J Oral Sci*. Forthcoming 2015.
2. Inaba M, Okuno S, Kumeda Y, Yamakawa T, Ishimura E, Nishizawa Y. Increased incidence of vertebral fracture in older female hemodialyzed patients with type 2 diabetes mellitus. *Calcif Tissue Int* 2005;76:256-60.
3. Epstein S, Leroith D. Diabetes and fragility fractures - a burgeoning epidemic? *Bone* 2008;43:3-6.
4. Lu H, Kraut D, Gerstenfeld LC, Graves DT. Diabetes interferes with the bone formation by affecting the expression of transcription factors that regulate osteoblast differentiation. *Endocrinology* 2003;144:346-52.
5. Katayama Y, Akatsu T, Yamamoto M, Kugai N, Nagata N. Role of nonenzymatic glycosylation of type I collagen in diabetic osteopenia. *J Bone Miner Res* 1996;11:931-7.
6. Sanguineti R, Storace D, Monacelli F, Federici A, Odetti P. Pentosidine effects on human osteoblasts in vitro. *Ann N Y Acad Sci* 2008;1126:166-72.
7. Yamamoto T, Ozono K, Miyauchi A, Kasayama S, Kojima Y, Shima M, et al. Role of advanced glycation end products in adynamic bone disease in patients with diabetic nephropathy. *Am J Kidney Dis* 2001;38:S161-4.
8. Tang SY, Zeenath U, Vashishth D. Effects of non-enzymatic glycation on cancellous bone fragility. *Bone* 2007;40:1144-51.
9. Roy B. Biomolecular basis of the role of diabetes mellitus in osteoporosis and bone fractures. *World J Diabetes* 2013;4:101-13.
10. Morikawa D, Nojiri H, Saita Y, Kobayashi K, Watanabe K, Ozawa Y, et al. Cytoplasmic reactive oxygen species and SOD1 regulate bone mass during mechanical unloading. *J Bone Miner Res* 2013;28:2368-80.
11. Schröder K. NADPH oxidases in bone homeostasis and osteoporosis. *Cell Mol Life Sci* 2015;72:25-38.
12. Mackenzie RM, Salt IP, Miller WH, Logan A, Ibrahim HA, Degasperis A, et al. Mitochondrial reactive oxygen species enhance AMP-activated protein kinase activation in the endothelium of patients with coronary artery disease and diabetes. *Clin Sci (Lond)* 2013;124:403-11.
13. Galli C, Piemontese M, Lumetti S, Manfredi E, Macaluso GM, Passeri G. The importance of WNT pathways for bone metabolism and their regulation by implant topography. *Eur Cell Mater* 2012;24:46-59.
14. Burgers TA, Williams BO. Regulation of Wnt/ β -catenin signaling within and from osteocytes. *Bone* 2013;54:244-9.
15. Poole KE, van Bezooijen RL, Loveridge N, Hamersma H, Papapoulos SE, Löwik CW, et al. Sclerostin is a delayed secreted product of osteocytes that inhibits bone formation. *FASEB J* 2005;19:1842-4.
16. Hofbauer LC, Brueck CC, Singh SK, Dobnig H. Osteoporosis in patients with diabetes mellitus. *J Bone Miner Res* 2007;22:1317-28.
17. Taut AD, Jin Q, Chung JH, Galindo-Moreno P, Yi ES, Sugai JV, et al. Sclerostin antibody stimulates bone regeneration after experimental periodontitis. *J Bone Miner Res* 2013;28:2347-56.
18. Ren Y, Han X, Ho SP, Harris SE, Cao Z, Economides AN, et al. Removal of SOST or blocking its product sclerostin rescues defects in the periodontitis mouse model. *FASEB J*. Forthcoming 2015.
19. Chen H, Xu X, Liu M, Zhang W, Ke HZ, Qin A, et al. Sclerostin antibody treatment causes greater alveolar crest height and bone mass in an ovariectomized rat model of localized periodontitis. *Bone* 2015;76:141-8.
20. Baek K, Hwang HR, Park HJ, Kwon A, Qadir AS, Ko SH, et al. TNF- α upregulates sclerostin expression in obese mice fed a high-fat diet. *J Cell Physiol* 2014;229:640-50.
21. Ardawi MS, Akhbar DH, Alshaikh A, Ahmed MM, Qari MH, Rouzi AA, et al. Increased serum sclerostin and decreased serum IGF-1 are associated with vertebral fractures among postmenopausal women with type-2 diabetes. *Bone* 2013;56:355-62.
22. García-Martín A, Rozas-Moreno P, Reyes-García R, Morales-Santana S, García-Fontana B, García-Salcedo JA, et al. Circulating levels of sclerostin are increased in patients with type 2 diabetes mellitus. *J Clin Endocrinol Metab* 2012;97:234-41.
23. Neumann T, Hofbauer LC, Rauner M, Lodes S, Kästner B, Franke S, et al. Clinical and endocrine correlates of circulating sclerostin levels in patients with type 1 diabetes mellitus. *Clin Endocrinol (Oxf)* 2014;80:649-55.
24. Jun JH, Lee SH, Kwak HB, Lee ZH, Seo SB, Woo KM, et al. N-acetylcysteine stimulates osteoblastic differentiation of mouse calvarial cells. *J Cell Biochem* 2008;103:1246-55.
25. Lee KS, Kim HJ, Li QL, Chi XZ, Ueta C, Komori T, et al. Runx2 is a common target of transforming growth factor beta1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. *Mol Cell Biol* 2000;

-
- 20:8783-92.
26. Kim JH, Lee DE, Woo GH, Cha JH, Bak EJ, Yoo YJ. Osteocytic sclerostin expression in alveolar bone in diabetic rats with ligature induced-periodontitis. *J Periodontol* 2015;1-14.
27. Tanaka K, Yamaguchi T, Kanazawa I, Sugimoto T. Effects of high glucose and advanced glycation end products on the expressions of sclerostin and RANKL as well as apoptosis in osteocyte-like MLO-Y4-A2 cells. *Biochem Biophys Res Commun* 2015;461:193-9.
28. Ahrens B. Antibodies in metabolic diseases. *N Biotechnol* 2011; 28:530-7.
29. Venieratos PD, Drossopoulou GI, Kapodistria KD, Tsilibary EC, Kitsiou PV. High glucose induces suppression of insulin signalling and apoptosis via upregulation of endogenous IL-1beta and suppressor of cytokine signalling-1 in mouse pancreatic beta cells. *Cell Signal* 2010;22:791-800.
30. David JP, Schett G. TNF and bone. *Curr Dir Autoimmun* 2010;11: 135-44.
31. Kawai VK, Stein CM, Perrien DS, Griffin MR. Effects of anti-tumor necrosis factor α agents on bone. *Curr Opin Rheumatol* 2012;24: 576-85.
32. Gilbert L, He X, Farmer P, Boden S, Kozlowski M, Rubin J, et al. Inhibition of osteoblast differentiation by tumor necrosis factor-alpha. *Endocrinology* 2000;141:3956-64.
33. Nakase T, Takaoka K, Masuhara K, Shimizu K, Yoshikawa H, Ochi T. Interleukin-1 beta enhances and tumor necrosis factor-alpha inhibits bone morphogenetic protein-2-induced alkaline phosphatase activity in MC3T3-E1 osteoblastic cells. *Bone* 1997;21:17-21.