치의과학석사학위논문

Effect of Hyperglycemia on the Expression of Sclerostin in Osteoblastic Cells

고농도의 포도당이 조골세포 계열세포의 sclerostin 유전자 발현에 미치는 영향

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서울대학교 대학원

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강 지 호

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위 원 장 _____ (인)

부위원장 _____ (인)

위 원 _____ (인)

Abstract

Effect of Hyperglycemia on the Expression of Sclerostin in Osteoblastic Cells

Jiho Kang
Department of Molecular Genetics
The Graduate School
Seoul National University
(Directed by Prof. Jeong-Hwa Baek, D.D.S., Ph.D.)

Sclerostin is an inhibitor of Wnt/β-catenin signaling and exerts negative effects on bone formation. Hyperglycemia is a representative symptom of diabetes mellitus. Recent studies demonstrated that osteoporotic fracture risk and serum sclerostin level is increased in diabetic patients. However, the molecular mechanism how sclerostin expression is enhanced in diabetic patients remains elusive. Therefore, in this study, the effect of hyperglycemia on the expression of sclerostin in the osteoblast lineage cells was examined. C2C12 cells were cultured in osteogenic medium (DMEM supplemented with 50 ng/ml BMP2 and 5% FBS). Osteocytic MLO-Y4 cells were cultured in αMEM supplemented with 5% calf serum and 5% FBS. To examine the effect of hyperglycemia, glucose concentration in culture medium was adjusted to 40 or 100 mM (*vs* 5 mM in control medium). Expression of sclerostin was examined by quantitative RT-PCR, Western blot and *Sost* promoter reporter assays. Top-

Flash reporter was used to examine the transcriptional activity of β-catenin/Tcf/Lef complex. Reactive oxygen species (ROS) production was examined by using DCF-DA reagent. Tumor necrosis factor α (TNFα) expression was examined by ELISA and quantitative RT-PCR. TNFα knockdown was induced by transient transfection of cells with TNFα siRNAs. Effect of ROS on sclerostin expression was examined by treating cells with 1 μM H_2O_2 or 20 mM N-acetylcysteine. High glucose concentration in culture medium increased the mRNA and protein levels of sclerostin and luciferase activity of *Sost* promoter reporter in both C2C12 and MLO-Y4 cells. High glucose suppressed Wnt3a-induced Top-Flash reporter activity and expression levels of osteoblast marker genes in C2C12 cells. High glucose increased ROS production and TNFα expression levels. Treatment of cells with H_2O_2 also enhanced expression levels of TNFα and sclerostin. In addition, N-acetylcysteine treatment or knockdown of TNFα attenuated high glucose-induced sclerostin expression. These results suggest that hyperglycemia increase sclerostin expression via increasing the production of ROS and TNFα.

Keywords: Hyperglycemia, sclerostin, osteocyte, ROS, TNFα

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Contents

Introduction

It is estimated by World Health Organization that 366 million people had diabetes mellitus (DM) in 2011 and this would have risen to 552 million till 2030 (Olokoba et al., 2012). Type 2 DM is the most common form of diabetes, characterized by hyperglycemia, insulin resistance and relative insulin deficiency. Recent studies demonstrated that osteoporotic fracture risk is increased in DM patients. Hip fracture risk was increased in type 1 DM patients and the risk of foot and hip fractures was increased in type 2 DM patients (Inaba et al., 2005).

Several mechanisms have been suggested to explain increased bone fracture risk in DM patients. Abnormal calcium 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 lower BMD in DM patients (Epstein et al., 2008). Chronic complications, including nephropathy, neuropathy, retinopathy and cardiomyopathy, occur in DM patients who have the damages to the blood vessels over time from hyperglycemia (Esposito et al., 2002). Microvascular disease makes blood supply to the bones decreased BMD (Smith, 1996). Diabetic animals produce sufficient amounts of immature mesenchymal tissue but fail to adequately

express the Runx2 and Dlx5, the transcription factors for osteoblast differentiation (Lu et al., 2003). Advanced glycation end products (AGEs) have been implicated in the development of DM-related osteopenia. In DM patients, higher 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 (Katayama et al., 1996; Sanguineti et al., 2008; Yamamoto et al., 2001). AGEs have also been suggested as a biomarker for the increased risk of fractures because AGEs are shown to be associated with bone rigidity (Tang et al., 2007). The receptor for AGEs (RAGE) is expressed in bone cells. Activation of RAGE stimulates the production of reactive oxygen species (ROS) and activate NF-κB signaling pathway (Roy et al., 2013). Intracellular ROS-mediated oxidative stress plays a crucial role in bone health. ROS promotes RANKL-mediated osteoclast differentiation and function (Su et al., 2011). Increased ROS-mediated oxidative stress in osteoblasts and osteocytes contributes to cell death of osteocytes and mechanical unloading-induced bone loss (Morikawa et al., 2013; Schröder, 2015). Patients with type 2 DM have shown elevated levels of mitochondrial ROS, which contribute to elevated osteolysis and bone fractures in DM patients (Mackenzie et al., 2013). Multiple factors, including negative calcium balance, microvascular damage, AGEs and ROS, may contribute to increased fracture risk in DM patients, but further studies are necessary to clarify the mechanisms involved in DM-associated with osteopenia and fracture risk.

Canonical Wnt signaling pathway controls cell proliferation and differentiation by regulating degradation and localization of β-catenin. Binding of canonical Wnt ligands to Frizzled and low-density lipoprotein receptor related protein (LRP5/6) inactivates β-catenin degradation complexes composed of Axin, glycogen synthase kinase 3β, casein kinase 1 and APC, resulting in accumulation and nuclear translocation of β-catenin. In the nucleus, β-catenin induces transcriptional activation of target genes by forming complex with lymphoid enhanced factor/T cell factor (LEF/TCF). Canonical Wnt signaling promotes differentiation of mesenchymal progenitor cells into osteoblasts. In osteoblasts, Wnt pathway induces cell proliferation, differentiation and matrix mineralization, whereas it blocks osteoclastogenesis by increasing the ratio of osteoprotegerin/RANKL (Suzuki K. et al. 2005). Sclerostin acts as an antagonist to Wnt signaling by binding to LRP5/6 co-receptors and inhibiting the binding of Wnt ligands (Burgers and Williams, 2013). Sclerostin is mainly produced by osteocytes (Poole et al., 2005). Sclerostin-mediated inhibition of Wnt signaling affects osteoblast differentiation and bone formation (Hofbauer et al., 2012). Serum sclerostin levels increase with age and further increase in postmenopausal women (Linares et al., 2007). Previous report demonstrated that serum sclerostin levels were higher in high fat diet-fed mice than control mice and that tumor necrosis factor α (TNFα) mediates the induction of sclerostin expression in high fat diet-fed mice (Baek et al., 2014). Compared to non-diabetic group, serum sclerostin levels are significantly higher in

both type 1 and type 2 DM patients independently of gender and age (Ardawi et al., 2013; García-Martín et al., 2012; Neumann et al., 2014; Zhou et al., 2013). These reports suggest that increased sclerostin expression contributes to DM-associated osteopenia and/or increased bone fracture risk. However, the molecular mechanisms involved in DM-mediated induction of sclerostin expression remain elusive. In this study, it was investigated how hyperglycemia regulates the expression of sclerostin in osteoblasts and osteocytes using murine cell lines C2C12 and MLO-Y4 cells.

Materials and Methods

Materials

The easy-BLUE™ and StarTaq™ reagents were ordered from iNtRON Biotechnology (Sungnam, Korea) and the AccuPower RT-Pre Mix was purchased from Bioneer (Daejeon, Korea). Easy BLUE RNA isolation kit was purchased from iNtRON Biotechnology (Sungnam, Korea). The SYBR Pre mix ExTaq™ was purchased from TaKaRa (Otsu, Japan). Tissue culture sera were purchased from HyClone (Logan, UT, USA). A Bright-Glo luciferase assay kit was obtained from Promega (Madison, WI, USA). Recombinant bone morphogenetic protein 2 (BMP2) was purchased from R&D Systems (Minneapolis, MN, USA) and added to culture medium in a concentration of 50 ng/ml. Wnt3a was purchased from R&D Systems and added to culture medium in a concentration of 50 ng/ml. To confirm the osteogenic differentiation, The Alkaline Phosphatase (ALP) staining kit was purchased from Sigma (St. Louis, MO, USA) and ALP staining was performed according to the manufacturer's instructions.

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 μg/ml streptomycin. After 2 days of confluence, culture medium was changed with DMEM supplemented with 50 ng/ml BMP2, 5% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin to induced osteogenic differentiation. Osteocytic MLO-Y4 cells were cultured in alpha modified Eagle's medium (αMEM) supplemented with 5% calf serum (BioWhittaker, Walkersville, MD, USA) and 5% FBS. *D*-(+)-Glucose (Sigma) was dissolved in DNase, RNase and protease free-sterile purified water and added to culture medium to obtain the indicated concentrations.

Reverse transcription-polymerase chain reaction (RT-PCR)

Quantitative RT-PCR was performed to examine mRNA expression levels. Total RNA was isolated by using an easyBLUE reagent and cDNA was synthesized from total RNA using the AccuPower RT PreMix and subsequently used for quantitative real time-PCR amplification using SYBR premix EX Taq. Mouse genes and the sequences of the PCR primers used for real-time PCR are shown in Table I.

Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used as the reference to normalize each sample for quantification.

Western blot analysis

For the whole cell lysate preparation, cells were lysed in protein extraction solution, PRO-PREP (iNtRON Biotechnology) which contains protease inhibitors such as 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 $\times$ *g* for 10 min, and the supernatants were used for western blot analysis. Each sample containing equal amounts of protein was subjected to SDS-PAGE. The proteins separated in the gel were subsequently transferred onto a PVDF membrane. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween20, incubated with sclerostin or actin antibody, and subsequently incubated with HRP-conjugated secondary antibody. Immune complexes were visualized using the Supex reagent and luminescence was detected with a LAS1000 (Fuji PhotoFilm; Tokyo, Japan).

Enzyme-linked immunosorbent assay (ELISA)

Cells were incubated for 48 h in the medium supplemented with high glucose or H_2O_2 and cell lysates were prepared as described above. The lysates were then sonicated briefly, centrifuged at 16,000 rpm for 10 min, and the supernatants were obtained. Expression levels of TNFα was determined using commercial ELISA kit (Koma Biotech, Seoul, Korea) according to the manufacturer's instructions.

Knockdown of TNFα

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

Luciferase reporter assays

The effect of high glucose on sclerostin transcription and transcriptional activity of β-catenin was examined by luciferase reporter assays. Construction of sclerostin promoter reporter (Sost-luc)

was previously described (Baek et al., 2014). For the transient transfection of plasmids, Lipofectamine reagent was used (Invitrogen, Carlsbad, CA, USA). In each transfection, 0.2 μg of reporters (Sost-luc, Top-Flash reporter) were used. When indicated, cells were transfected with 0.2 μg of β-catenin expression plasmid. Cells were then incubated for 24 or 48 h in the presence or absence of high glucose. Luciferase activity was measured using the Bright-Glo luciferase assay kit according to the manufacturer's instructions.

DCF-DA assay for measuring intracellular ROS production

Assays were performed as described previously (Arnold et al., 2001) with modifications. Cells were grown to 100% confluency and incubated in the presence of 2 μM DCF-DA in the dark at room temperature for 10 min. Then emitted fluorescence was measured at 490nm and this value was used as a baseline for the following experiments. Then cells were incubated in the presence or absence of high glucose, TNFα or H_2O_2 for the indicated periods.

Statistical analysis

The data were presented as the mean±SD. The statistical significance of the results was assessed by Student's *t*-test. A P-value less than 0.05 was considered statistically significant.

Table 1. Primer sequences for quantitative RT-PCR

Genes	Forward (5’ to 3’)	Reverse (5’ to 3’)
Runx2	TTCTCCAACCCACGAATGCAC	CAGGTACGTGTGGTAGTGAGT
Alp	CCAACTCTTTTGTGCCAGAGA	GGCTACATTGGTGTTGAGCTTTT
Ocn	CTGACAAAGCCTTCATGTCCAA	GCGCCGGAGTCTGTTCACTA
Sost	AGCCTTCAGGAATGATGCCAC	CTTTGGCGTCATAGGGATGGT
TNF α	GACGACAGCAAGGGACTAGC	GCTTTCTGTGCTCATGGTGC
Gapdh	TCACCATCTTCCAGGAGCG	CTGCTTACCACCTTCTTGA

Results

High glucose increases sclerostin expression in C2C12 cells

C2C12 is a mouse myoblast cell line derived from the thigh muscle of C3H mice after a crush injury. C2C12 cells can be differentiated into osteoblasts by treatment with BMP2 (Lee et al., 2000). To examine the effect of high glucose on the expression levels of sclerostin, osteoblastic differentiation of C2C12 cells was induced by incubation of confluent cells in the presence of 50 ng/ml BMP2. With the initiation of the osteogenic differentiation, C2C12 cells were exposed to varying concentrations of high glucose (40-100 mM) for 48 h. Quantitative RT-PCR results demonstrated that high glucose significantly enhanced mRNA levels of sclerostin in all of the concentration examined (Fig. 1A). However, western blot analysis showed that increase in sclerostin protein levels was most significant in 100 mM glucose (Fig. 1B). Therefore, following experiments were performed using the glucose concentration of 100 mM. High glucose-induced sclerostin expression was also observed in C2C12 cells after incubation for 24 h (Fig. 1C, 1D). Promoter reporter assay using Sost-luc showed that high glucose increased *Sost* promoter activity, suggesting that high glucose increased transcription of *Sost* gene (Fig. 1E).

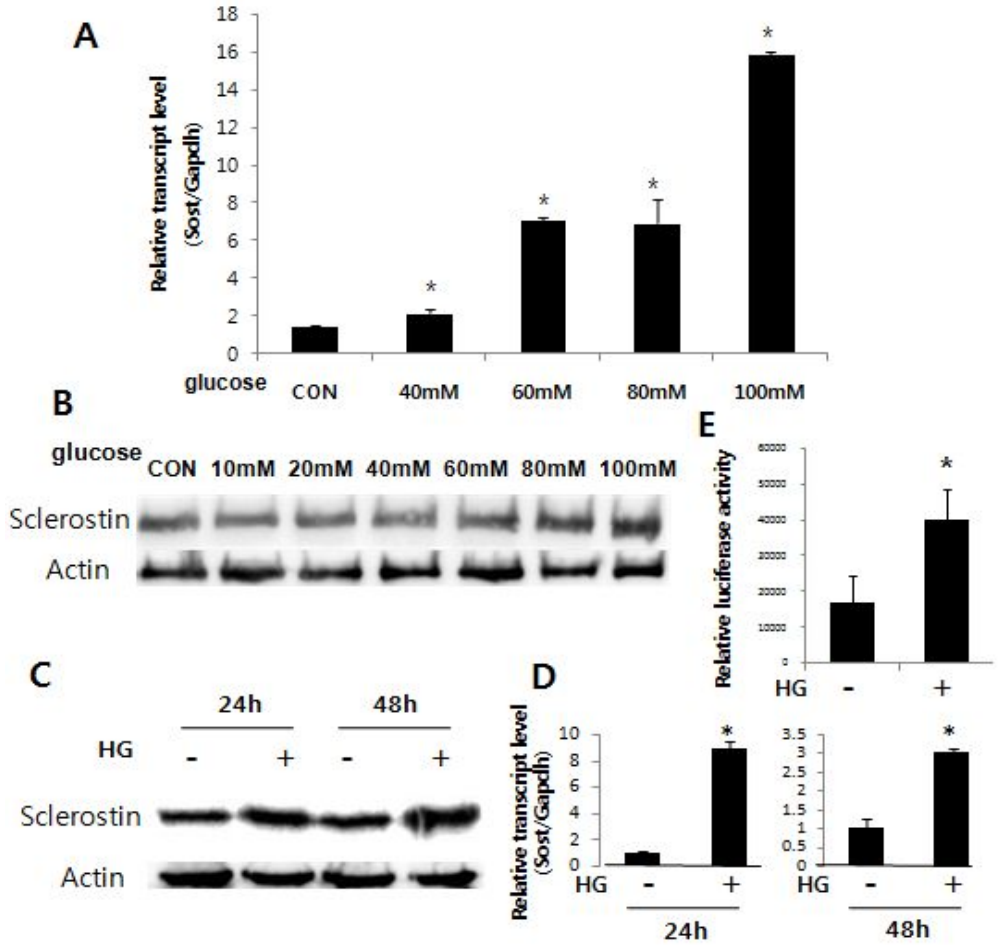

Fig. 1. High glucose increases sclerostin expression in C2C12 cells. (A, B) C2C12 cells were cultured in osteogenic medium in the presence or absence of high glucose for 48 h, and RT-PCR (A) and western blot analysis (B) was performed. (C, D) C2C12 cells were cultured in the presence or absence of high glucose (100 mM) for 24 or 48 h, and RT-PCR (C) and western blot analysis (D) was performed. (E) C2C12 cells were transiently transfected with reporter plasmids containing *Sost* promoter and incubated in the presence or absence of high glucose for 24 h. Subsequently luciferase reporter assays were performed and data were presented as relative luciferase activity. Quantitative data were presented as the mean±SD (*$p<0.05$, compared to control). HG, glucose 100 mM

High glucose inhibits Wnt/β-catenin signaling in C2C12 cells

Because high glucose increased expression levels of sclerostin in C2C12 cells, it was next examined whether high glucose suppresses Wnt/β-catenin signaling in C2C12 cells. C2C12 cells were incubated for 24 h in the presence or absence of Wnt3a or 100 mM glucose and expression levels of osteogenic marker genes, including ALP, Runx2, osteocalcin and osterix, were examined. High glucose significantly suppressed mRNA expression levels of Wnt3a-induced marker genes (Fig. 2A). To further clarify the inhibitory effect of high glucose on Wnt/β-catenin signaling, Top-Flash luciferase assay was performed. Wnt3a increased Top-Flash activity and high glucose significantly suppressed Wnt3a-induced reporter activity (Fig. 2A). These results suggest that high glucose-induced sclerostin inhibits Wnt/β-catenin signaling in C2C12 cells.

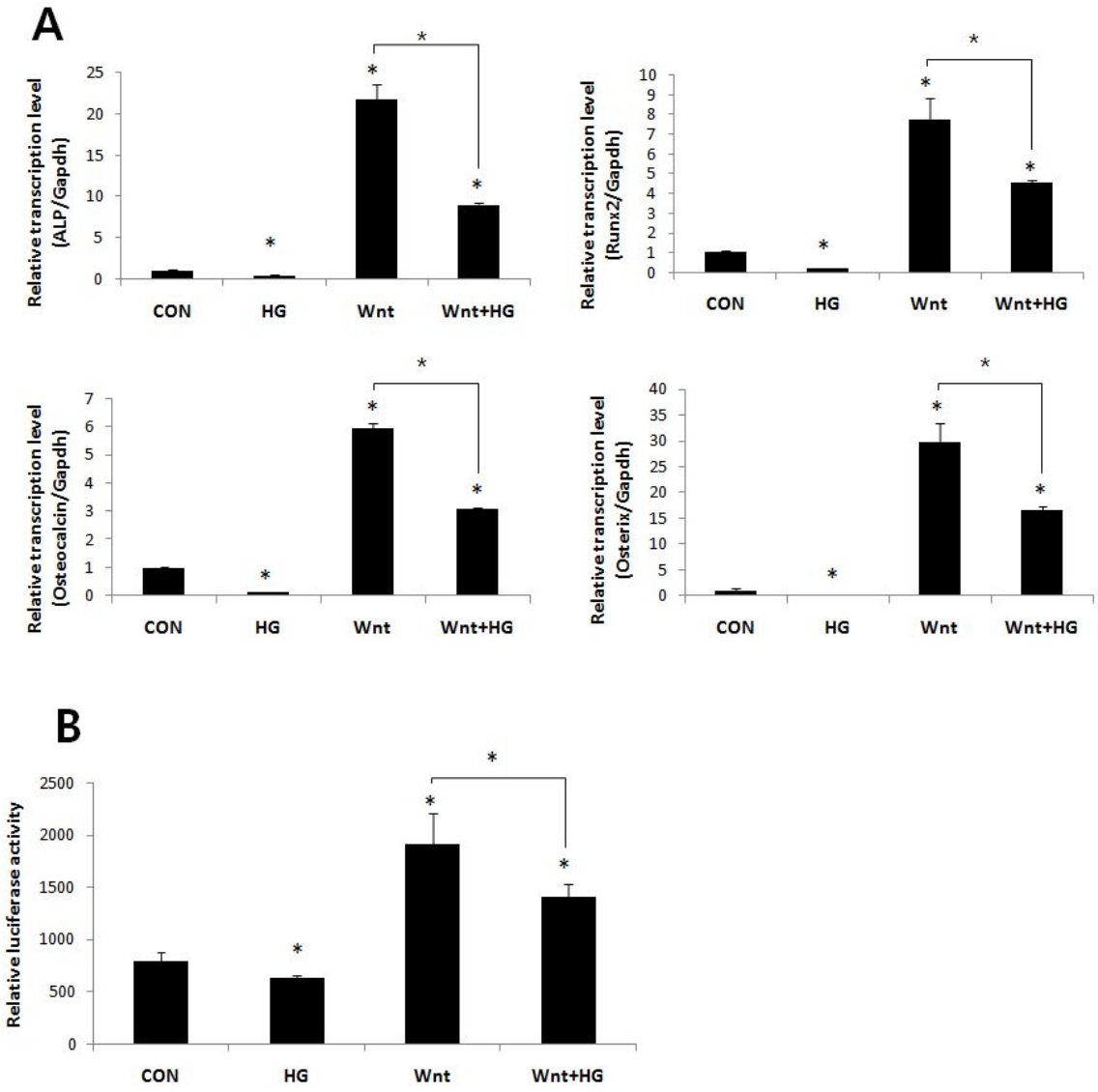

Fig. 2. High glucose negatively regulates Wnt/β-catenin signaling in C2C12 cells. (A) C2C12 cells were incubated for 24 h in the presence or absence of Wnt3a (50 ng/ml) or high glucose (100 mM), followed by quantitative RT-PCR of osteogenic marker genes. (B) C2C12 cells were transiently transfected with β-catenin expression plasmid and Top-Flash luciferase reporter vector and cultured in the presence or absence of Wnt3a or high glucose (40 mM) for 72 h. Data represent the mean±SD (*$p<0.05$).

High glucose-induced ROS production contributes to enhanced sclerostin expression in C2C12 cells

It was next examined whether high glucose enhances production of ROS in C2C12 cells. Intracellular ROS levels were determined using DCF-DA. H_2O_2 (1 mM) was used as a positive control. Compared to control group, ROS production was significantly higher in high glucose and H_2O_2 groups (Fig. 3A). Then it was examined whether increased ROS level contributes to sclerostin expression. Treatment of C2C12 cells with 0.1 to 1 mM H_2O_2 for 48 h significantly induced mRNA expression of sclerostin (Fig. 3B). To further clarify the effect of ROS on sclerostin expression, it was examined whether N-acetylcysteine (20 mM), an antioxidant, can block the effect of high glucose and H_2O_2 on sclerostin expression. Addition of N-acetylcysteine significantly suppressed high glucose- and H_2O_2–induced sclerostin expression in both mRNA and protein levels (Fig. 3C). These results suggest that high glucose-induced ROS production contributes to induction of sclerostin expression in C2C12 cells.

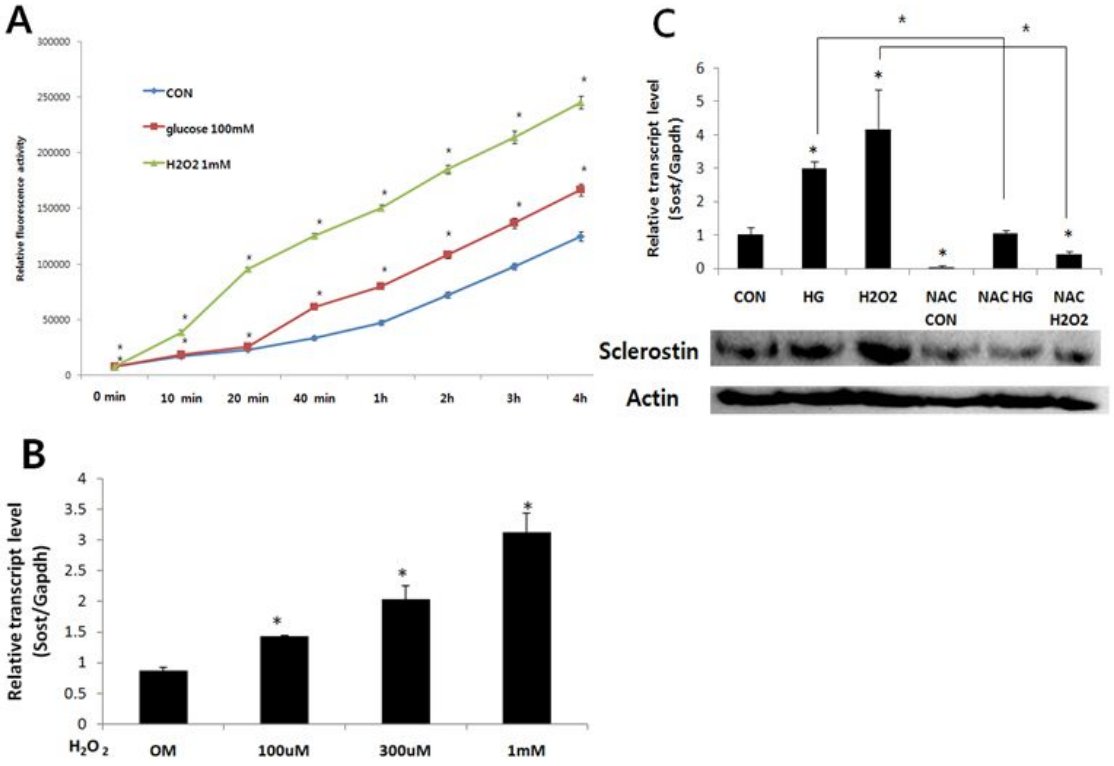

Fig. 3. High glucose-induced ROS production contribute to sclerostin expression in C2C12 cells. (A) DCF-DA was added to culture medium and cells were treated with high glucose (100 mM) or H_2O_2 for the indicated periods, followed by measuring fluorescence from oxidized DCF. (B) C2C12 cells were incubated for 48 h in the presence of H_2O_2 of the indicated concentrations, followed by quantitative RT-PCR for sclerostin. (C) C2C12 cells were incubated for 48 h in the presence or absence of indicated reagents, followed by quantitative RT-PCR and western blot analysis. HG, 100 mM High glucose ; H_2O_2, 1 mM; NAC, 20 mM N-acetylcysteine.

High glucose-induced TNFα expression contributes to enhanced sclerostin expression in C2C12 cells

Because the previous report demonstrated that TNFα enhances sclerostin expression in MLO-Y4 cells (Baek et al., 2014), it was examined whether high glucose stimulates TNFα expression in C2C12 cells. High glucose significantly increased mRNA expression levels of TNFα (Fig. 4A). H_2O_2 also upregulated sclerostin mRNA levels (Fig. 4B). High glucose- and H_2O_2-induced TNFα expression was also observed in the protein levels (Fig. 4C). Because H_2O_2 showed stimulatory effect on TNFα expression, it was examined whether antioxidant treatment attenuate high glucose-induced TNFα expression. Addition of N-acetylcysteine significantly downregulated high glucose- and H_2O_2-induced TNFα expression (Fig. 4D), suggesting that ROS is involved in high-glucose induced TNFα expression.

To examine whether TNFα mediates high glucose-induced sclerostin expression, knockdown of TNFα was induced using TNFα siRNA. Quantitative RT-PCR results showed that TNFα siRNA suppressed high glucose- and H_2O_2-induced TNFα expression (Fig. 4E, upper panel). Compared to control siRNA-transfected cells, TNFα siRNA-transfected cells did not show induction of sclerostin

expression by high glucose and H_2O_2 (Fig. 4E, middle and lower panels). These results suggest that high glucose-induced ROS increase TNFα expression, which subsequently enhance sclerostin expression in C2C12 cells.

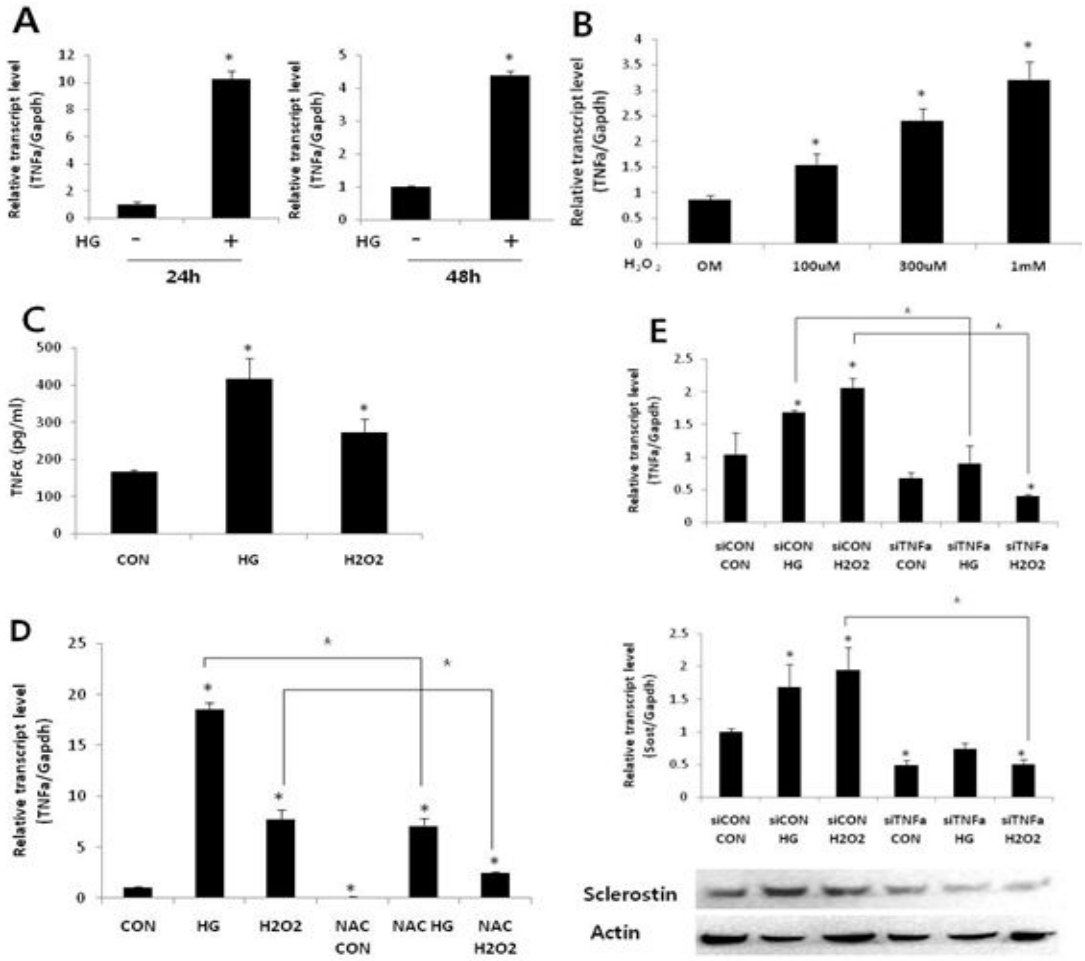

Fig. 4. TNFα mediates high glucose-induced sclerostin expression in C2C12 cells. (A) C2C12 cells were cultured in the presence or absence of high glucose (100 mM) for 24 or 48 h, and RT-PCR for TNFα was performed. (B) C2C12 cells were incubated for 48 h in the presence of H_2O_2 of the indicated concentrations, followed by quantitative RT-PCR for TNFα. (C) C2C12 cells were incubated for 48 h in the presence of the indicated reagents, followed by ELISA assay for TNFα using cell lysates. (D) C2C12 cells were incubated for 48 h in the presence or absence of indicated reagents, followed by quantitative RT-PCR. (E) C2C12 cells were transiently transfected with control siRNA or TNFα siRNA, followed by incubation for 48 h in the presence or absence of the indicated reagents. Then quantitative RT-PCR (upper and middle panels) and western blot analysis (lower panel) was performed.

High glucose increases sclerostin expression in MLO-Y4 cells

Because sclerostin is mainly expressed in osteocytes, the effect of high glucose on sclerostin expression was also examined using MYO-Y4 osteocytic cell line. Similar to the results obtained from C2C12 cells, high glucose significantly increased sclerostin expression in both mRNA and protein levels (Fig. 5A, 5B). Furthermore, luciferase reporter assay using Sost-luc vector demonstrated high glucose significantly enhanced promoter activity of *Sost* gene (Fig. 5C), suggesting high glucose increases transcription of *Sost* gene. Top-Flash reporter assay result also showed that high glucose significantly inhibited Wnt3a/β-catenin-induced luciferase activity (Fig. 5D). These results indicate that high glucose increases sclerostin expression in osteocytes.

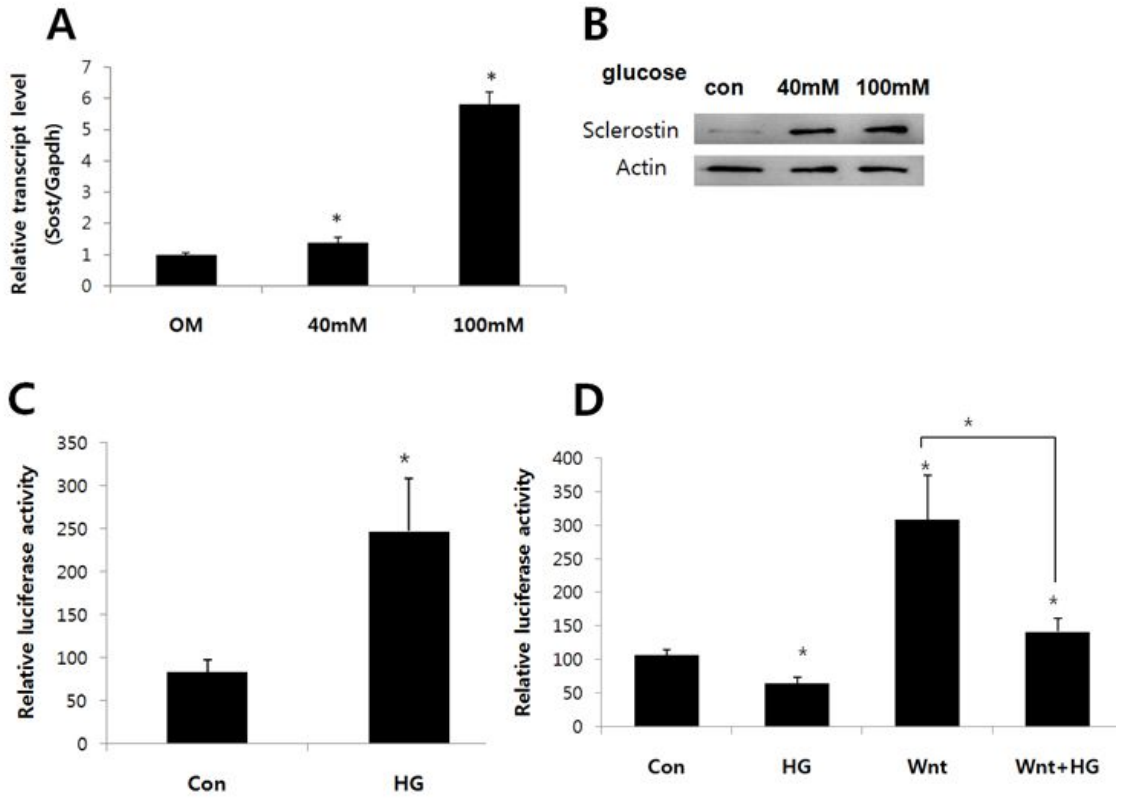

Fig. 5. High glucose increases sclerostin expression in MLO-Y4 cells. (A, B) MLO-Y4 cells were incubated for 48 h in the presence or absence of high glucose treatment, followed by quantitative RT-PCR (A) and western blot analysis (B). (C) MLO-Y4 cells were transiently transfected with reporter plasmids containing *Sost* promoter and incubated in the presence or absence of high glucose (100 mM) for 24 h. Subsequently luciferase reporter assays were performed. (D) MLO-Y4 cells were transiently transfected with β-catenin expression plasmid and Top-Flash luciferase reporter vector and cultured in the presence or absence of Wnt3a (50 ng/ml) or high glucose (40 mM) for 72 h. Subsequently, luciferase activity was measured.

High glucose-induced ROS production and subsequent induction of TNFα expression mediates sclerostin expression in MLO-Y4 cells

It was next examined whether increased production of ROS and TNFα also mediates high glucose-induced sclerostin expression in MLO-Y4 cells. Incubation of MLO-Y4 cells in the presence of high glucose significantly increased ROS production (Fig. 6A). In addition, high glucose and H_2O_2 significantly increased TNFα expression in MLO-Y4 cells (Fig. 6B). Furthermore, N-acetylcysteine treatment significantly downregulated high glucose- and H_2O_2-induced expression of TNFα and sclerostin (Fig. 6C). TNFα knockdown suppressed high glucose- and H_2O_2-induced sclerostin expression (Fig. 6D). These results further confirmed that high glucose/ROS/TNFα axis is working to increase expression levels of sclerostin in MLO-Y4 cells.

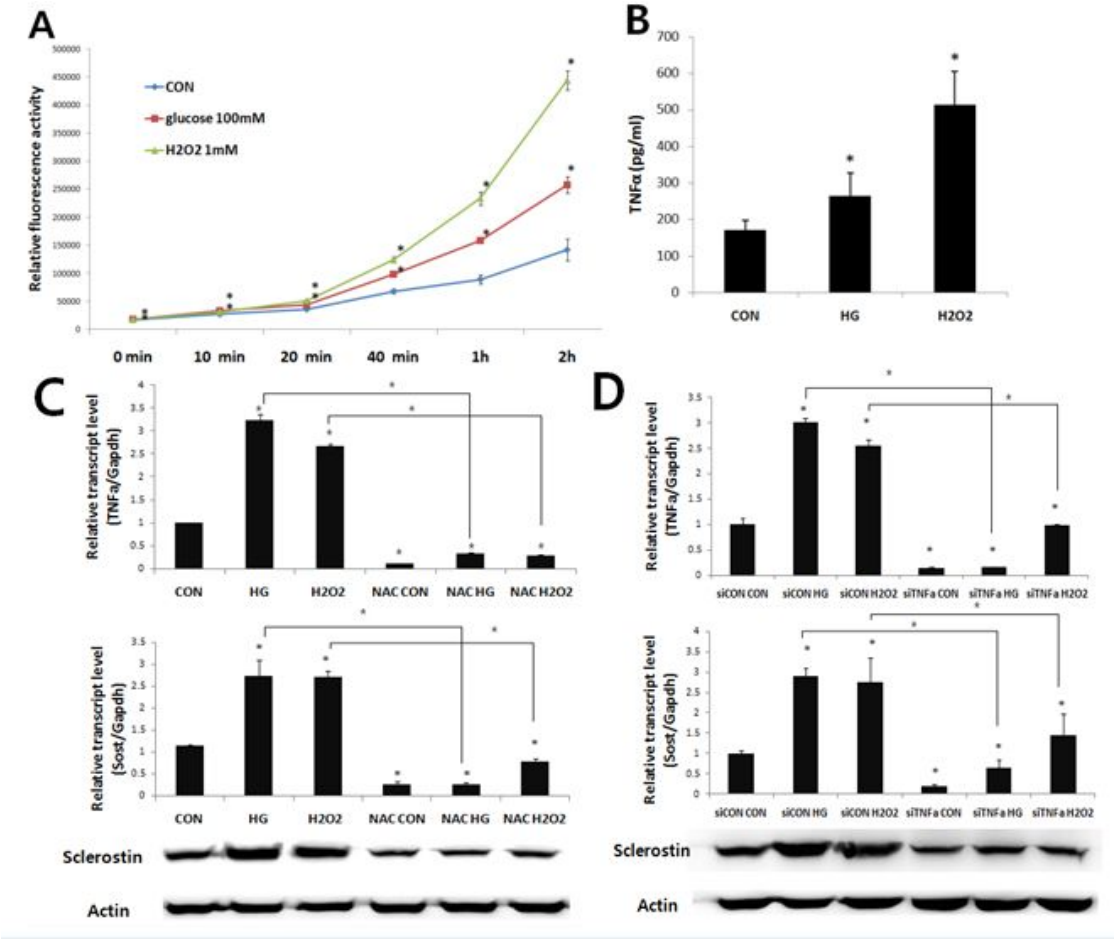

Fig. 6. ROS and TNFα mediates high glucose-induced sclerostin expression in MLO-Y4 cells. (A) DCF-DA was added to culture medium and MLO-Y4 cells were treated with high glucose or H_2O_2 for the indicated periods, followed by measuring fluorescence from oxidized DCF. (B) MLO-Y4 cells were incubated for 48 h in the presence of high glucose (100 mM) or H_2O_2 (1 mM), followed by ELISA assay for TNFα using cell lysates. (C) MLO-Y4 cells were incubated for 48 h in the presence or absence of indicated reagents, followed by quantitative RT-PCR and western blot analysis. (D) MLO-Y4 cells were transiently transfected with control siRNA or TNFα siRNA, followed by incubation for 48 h in the presence or absence of the indicated reagents. Then quantitative RT-PCR and western blot analysis was performed.

Discussion

Sclerostin is an inhibitor of Wnt/β-catenin signaling and exerts negative effects on bone formation. Hyperglycemia is a representative symptom of diabetes mellitus. Recent studies demonstrated that osteoporotic fracture risk and serum sclerostin level is increased in diabetic patients (Inaba et al., 2005) and that increased serum sclerostin was associated with vertebral fractures in type 2 DM patients (Yamamoto et al., 2013). However, the molecular mechanism how sclerostin expression is enhanced in diabetic patients remains elusive. In this study, it was demonstrated that exposure of osteoblast lineage cells to high concentration of glucose induces ROS and TNFα production, which subsequently upregulates expression levels of sclerostin.

Hyperglycemia is the representative symptom of DM. The chronic hyperglycemia can directly induce an inflammatory state, where the increment in cytokines can lead to degradation of the pancreatic beta cells and disorder of the endocrine pancreas in both type 1 and type2 DM (Ahrens et al., 2011). In DM patients, there is a significant increase in levels of inflammatory cytokines, including IL-6, IL-18, IL-1 and TNFα in the blood of patients (Venieratos et al., 2010). Similar to this *in vivo* phenomenon, exposure of C2C12 and MLO-Y4 cells to high glucose significantly increased the expression levels of TNFα mRNA and protein. Induction of TNFα expression by high

glucose was blocked by N-acetylcysteine, suggesting that ROS production is necessary to high glucose-induced TNFα expression in osteoblast lineage cells. TNFα plays an important role in inflammatory bone loss, including rheumatoid arthritis, periodontitis and osteoporosis (David and Schett, 2010; Kawai et al., 2012). TNFα also inhibits osteogenic differentiation and bone formation (Gilbert et al., 2000; Nakase et al., 1997). In addition, it was reported that TNFα enhances sclerostin expression in a NF-κB-dependent manner in osteocytes (Baek et al., 2014). Taken together, it is suggested that enhanced TNFα production by high glucose contributes to decreased BMD and/or increased fracture risk in DM patients.

In DM patients, higher amount of AGEs is accumulated. Activation of RAGE by AGEs stimulates the production of ROS and activate NF-κB signaling pathway (Roy et al., 2013). In the present study, high glucose increased ROS production in both C2C12 and MLO-Y4 cells, and the addition of antioxidant prevented high glucose-induced TNFα and sclerostin expression. Therefore, it is suggested that AGEs as well as hyperglycemia contribute to increase in serum sclerostin levels in DM patients via enhancing ROS production.

In conclusion, in this study, it was demonstrated that exposure of osteoblast lineage cells to high glucose enhanced ROS and TNFα production, which subsequently induced sclerostin expression. Therefore, it is suggested that regulation of TNFα and oxidative

stress levels in the diabetic patients may be of therapeutic relevance for the bone complications derived from chronic hyperglycemia.

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국문초록

고농도의 포도당이 조골세포 계열세포의 sclerostin 유전자 발현에 미치는 영향

서울대학교 대학원 분자유전학 전공

(지도교수 백정화)

강지호

Sclerostin은 Wnt/β-catenin 신호에 대한 억제제로 작용하여 골 형성에 부정적인 영향을 끼치는 분자로 알려져 있다. 당뇨는 특징적으로 고혈당 증상을 보이며 최근 연구 결과에 따르면 골다공증에 의한 골절위험률이 당뇨 환자에서 더 높게 나타나며, 당뇨환자의 혈중 sclerostin 수준이 정상인에 비해 높은 것으로 알려져 있으나 당뇨환자에서 sclerostin 발현이 증가되는 기전은 잘 알려져 있지 않다. 그러므로 본 연구에서는 고농도의 포도당이 조골세포 계열 세포의 sclerostin 발현에 미치는 영향 및 그 조절 기전을 알아보고자 하였다. C2C12 세포는 조골세포 분화배지(5% FBS, 50 ng/ml BMP2를 포함한 DMEM)에서 배양했다. 골세포인 MLO-Y4 세포는 5% FBS, 5% calf serum을 포함한 배지에서 배양했다. 배양액 내 포도당의 농도를 5 mM에서 40 또는 100 mM로 증가시켰을 때 *Sost* 유전자 발현 변화에 미치는 영향을 알아보기 위해 정량적 RT-PCR, Western blot, *Sost* 프로모터 리포터 분석을 시행했다. Top-Flash 리포터는 β-catenin/Tcf/Lef complex의 전사 활성 정도를 측정하기 위해 사용되었다. 세포에 DCF-DA 시약을 이용하여 세포 내 활성화산소종의 생성 정도를 확인하였다. 고농도 포도당에 의한 TNFα의 발현 변화는 ELISA와 정량적 RT-PCR 분석을

통해 확인하였다. TNFα siRNA를 이용하여 TNFα 발현 저해를 유도하였고, 세포내 활성화산소종의 발생을 촉진 또는 억제하기 위해 1 μM H_2O_2, 20 mM N-acetylcysteine을 각각 처리하였다. 배양액 내 포도당 농도를 40 또는 100 mM로 증가시킨 경우 C2C12, MLO-Y4 두 세포 모두에서 sclerostin mRNA와 단백질의 수준이 유의하게 증가하였고, *Sost* promoter reporter 활성이 증가하였다. 대조군과 비교하여 100 mM 포도당은 C2C12세포에서 Wnt3a에 의해 증가된 조골세포 분화표지의 발현수준을 유의하게 감소시켰으며, Wnt3a에 의한 Top-Flash 리포터 활성 증가를 억제하였다. 100 mM 포도당을 세포에 처리하면 활성화산소종 생성이 촉진되며 TNFα의 발현 수준을 증가시켰다. 또한 H_2O_2를 처리해준 경우 두 세포 모두에서 TNFα와 sclerostin 발현 수준이 증가되었고 이러한 현상은 N-acetylcysteine에 의해 차단되었다. TNFα siRNA를 이용하여 TNFα 발현을 억제한 세포에서는 고농도 포도당 및 H_2O_2에 의한 sclerostin 발현 증가가 차단되었다. 이상의 결과는 고혈당이 골세포의 sclerostin 발현 증가를 유도하며, 그 과정에 활성화산소종 생성 및 TNFα 발현 증가가 관여할 수 있음을 시사하였다.

핵심어: 고혈당, sclerostin, 골세포, 활성화산소종, TNFα

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