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

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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-alpha (TNFα) 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 TNFα expression levels. Treatment of cells with H2O2 also enhanced the expression levels of TNFα and sclerostin. In addition, N-acetylcysteine treatment or knockdown of TNFα attenuated high glucose-induced sclerostin expression.

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

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

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-
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Hypercglycemia increases sclerostin expression that tumor necrosis factor α (TNFα) 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-PreMix 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 μg/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 μg/mL streptomycin after two days of confluence. Osteocytic MLO-Y4 cells were cultured in alpha modified Eagle's medium (αMEM) 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′-AGCCTTCCAGGAATGATGCGAC-3′ and (r) 5′-CTTGGCGT-CATAGGGATGTT-3′; TNFa, (f) 5′-GACGACAGCAAGGGACTAGC-3′.
and (r) 5′-GCTTCTGTGCATGATGTC-3′; Runx2, (f) 5′-TTCTC-CAACCCACGGATGCC-3′ and (r) 5′-GATGTTGGTGTAAGTGCAG-3′; osteonectin, (f) 5′-CCACCATCCATCACTC-3′ and (r) 5′-CTTGACACACGAGCCAT-3′; alkaline phosphatase, (f) 5′-CCACTITGCGCCAG-3′ and (r) 5′-GGCTACATTGGTGTTGAGCT-3′; osteocalcin, (f) 5′-GCAAAAGGCTCCTATG-3′ and (r) 5′-GCCGGAGTCTGGTCCAC-3′; and GAPDH, (f) 5′-CTAGACACTTGTGCAAGC-3′ and (r) 5′-CCAGGTTTCTTACTCCTG-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 aprotonin (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 Supex 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 × 10^4 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 osteocyte 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.
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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 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. H₂O₂ (1 mM) was used as a positive control. Compared to the control cells, the ROS production was significantly higher in high glucose- and H₂O₂-treated cells (Fig. 3). The ROS levels in the control, 100 mM glucose, and 1 mM H₂O₂ groups after two hours of incubation were 141.75 ± 19.11, 257.52 ± 14.679, and 443.84 ± 16.675, respectively. In order to explore whether increased ROS levels contribute to sclerostin expression, C2C12 cells were incubated with 0.1–1 mM H₂O₂ for 48 hours and the expression levels of sclerostin were determined. Quantitative RT-PCR results demonstrated that H₂O₂ significantly increased sclerostin mRNA levels in a dose-depen-
dent manner (Fig. 4A). The fold changes of sclerostin mRNA in 0, 0.1, 0.3, and 1.0 mM H$_2$O$_2$ 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 H$_2$O$_2$–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 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 ± 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. Dichlorofluorescin diacetate was added to the culture medium, and (A) C2C12 and (B) MLO-Y4 cells were treated with high glucose (100 mM) or H$_2$O$_2$ (1 mM) for the indicated time periods, followed by measuring fluorescence from oxidized dichlorofluorescin. The graphs indicate the mean ± 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, dichlorofluorescin; CON, control; HG, high glucose; m, minutes; h, hours.
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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.

**TNFα contributes to high glucose-induced sclerostin expression**

Since a previous report has demonstrated that TNFα enhances sclerostin expression in MLO-Y4 cells [20], we examined whether high glucose stimulates TNFα expression. Both high glucose and H2O2 significantly increased the expression levels of the TNFα protein (Fig. 5A, B). Since H2O2 also exerted a stimulatory effect on TNFα expression, we examined whether antioxidant treatment attenuates high glucose-induced TNFα expression. The addition of N-acetylcysteine significantly downregulated high glucose- and H2O2-induced TNFα expression (Fig. 5C, D). High glucose-induced TNFα 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 TNFα 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 TNFα expression.

In order to further examine whether TNFα mediates high glucose-induced sclerostin expression, the knockdown of TNFα was induced using TNFα siRNA. Quantitative RT-PCR results showed that TNFα siRNA suppressed high glucose- and H2O2-induced TNFα expression (Fig. 6A–D). Compared to control siRNA-transfected cells, high glucose and H2O2-induced sclerostin expression was significantly attenuated in TNFα siRNA-transfected cells (Fig. 6A–D). High glucose-induced sclerostin mRNA levels in the control siRNA compared to TNFα 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 TNFα 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 TNFα expression, which subsequently enhances sclerostin expression.

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 (P<0.01, *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.
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 TNFα 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 TNFα expression was insignificant, suggesting that the regulatory effect of high glucose on sclerostin and TNFα expression is independent of osmolarity (data not shown). The stimulatory effect of high glucose on sclerostin 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 TNFα, 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 TNFα mRNA and protein. The induction of TNFα expression by high glucose was blocked by N-acetylcysteine, suggesting that ROS production is necessary for high glucose-induced TNFα expression. TNFα plays an important role in inflammatory bone loss, including rheumatoid arthritis, periodontitis, and osteoporosis [30,31]. TNFα also inhibits osteogenic differentiation and bone formation [32,33]. Since TNFα also enhances sclerostin expression in a NF-κB-dependent manner in osteocytes [20], it is suggested that enhanced TNFα production by high glucose contributes to increased alveolar bone loss in DM patients.

In DM patients, a greater amount of AGEs is accumulated. The
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Figure 6. TNFα mediates high glucose-induced sclerostin expression. (A, B, E) C2C12 and (C, D, F) MLO-Y4 cells were transiently transfected with control siRNA or TNFα siRNA, followed by incubation for 48 hours in the presence or absence of the indicated reagents. (A–D) Quantitative RT-PCR for TNFα and sclerostin, and (E, F) Western blot analysis were then performed. The graphs indicate the mean ± standard deviation of the triplicate samples (a) \( P < 0.05 \), (b) \( P < 0.01 \), (c) \( P < 0.001 \), (d) \( P < 0.0001 \). TNFα, tumor necrosis factor-alpha; 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-κB 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 TNFα 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 TNFα production, which subsequently induced sclerostin expression. Considering that TNFα 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 TNFα 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.
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