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Unique Cartilage Matrix-Associated Protein Alleviates Hyperglycemic Stress in MC3T3-E1 Osteoblasts

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Unique cartilage matrix-associated protein (UCMA) is an extrahepatic vitamin K-dependent protein rich in γ-carboxylated (Gla) residues. UCMA has been recognized for its ability to promote osteoblast differentiation and enhance bone formation; however, its impact on osteoblasts under hyperglycemic stress remains unknown. In this paper, we investigated the effect of UCMA on MC3T3-E1 osteoblastic cells under hyperglycemic conditions. After exposure to high glucose, the MC3T3-E1 cells were treated with recombinant UCMA proteins. CellROX and MitoSOX staining showed that the production of reactive oxygen species (ROS), which initially increased under high-glucose conditions in MC3T3-E1 cells, decreased after UCMA treatment. Additionally, quantitative polymerase chain reaction revealed increased expression of antioxidant genes, nuclear factor erythroid 2-related factor 2 and superoxide dismutase 1, in the MC3T3-E1 cells exposed to both high glucose and UCMA. UCMA treatment downregulated the expression of heme oxygenase-1, which reduced its translocation from the cytosol to the nucleus. Moreover, the expression of dynamin-related protein 1, a mitochondrial fission marker, was upregulated, and AKT signaling was inhibited after UCMA treatment. Overall, UCMA appears to mitigate ROS production, increase antioxidant gene expression, impact mitochondrial dynamics, and modulate AKT signaling in osteoblasts exposed to high-glucose conditions. This study advances our understanding of the cellular mechanism of UCMA and suggests its potential use as a novel therapeutic agent for bone complications related to metabolic disorders.

Key words : Hyperglycemic stress, osteoblasts, ROS production, unique cartilage matrix-associated protein

Introduction

Osteoblasts are bone-forming cells differentiated from mesenchymal stem cells (MSCs) that reside in the bone marrow. The differentiation of MSCs into osteoblasts is elaborately regulated by essential transcription factors including runt-related transcription factor 2 (Runx2), osterix, and distal-less homeobox 5 (Dlx5) [11]. Upon differentiation, osteoblasts deposit and mineralize the bone matrix by secreting proteoglycans, glycoproteins, and γ-carboxylated (Gla) proteins, which are crucial for regulating adhesion, migration, proliferation, and differentiation of bone cells [3]. Osteoblasts are required for maintaining bone homeostasis [28] and use glucose as their primary energy source. Glucose is transported into cells through glucose transporter 1 (Glut1) and Glut3, depending on the intracellular glucose concentration [29, 32]. Although high glucose levels do not alter Glut1 expression in osteoblasts [4], they induce bone loss by decreasing the function of osteoblasts [34].

Hyperglycemia is characterized by high blood glucose levels and diverse physiological effects [2, 8, 19]. It increases the production of reactive oxygen species (ROS) and disrupts mitochondrial function [10]. In particular, in osteoblasts, it inhibits cell proliferation [9], enhances cell apoptosis [1], and decreases the expression of Runx2, Dlx5, and bone morphogenetic proteins, leading to reduced osteoblast differentiation and an increased number of immature osteoblasts [4]. Consequently, there is a gradual loss in the mineralization function

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of osteoblasts, causing bone loss. In addition, hyperglycemia-induced ROS production in osteoblasts affects mitochondrial function by disrupting the balance between mitochondrial fission and fusion [9, 10].

Unique cartilage matrix-associated protein (UCMA) was first discovered in cartilage and identified as one of the vitamin K-dependent proteins (VKDPs) [24, 26]. UCMA is γ-carboxylated by vitamin K-dependent γ-glutamyl carboxylase, resulting in an increased calcium-binding affinity [7]. It accelerates osteoblast differentiation and calcium deposition in the bone [14] and inhibits ectopic calcification in the vascular system [33]. Other extrahepatic VKDPs, matrix Gla protein (MGP) and osteocalcin (OC), also play important roles in calcification [24]. It has been reported that OC inhibits ROS production induced by high glucose in osteoblasts [18]. However, the potential effect of UCMA under high glucose conditions has not yet been elucidated. Therefore, we investigated the alleviating effect of UCMA on hyperglycemic stress regulation in MC3T3-E1 osteoblasts.

Materials and Methods

Cell culture

MC3T3-E1 osteoblastic cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in α-minimum essential medium (HyClone, Logan, UT, USA) containing the 5.5 mM glucose concentration, which was supplemented with 10% fetal bovine serum (HyClone), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Carlsbad, CA, USA) at 37℃ in an atmosphere of 5% CO_2. Next, the cells were seeded at a density of 2×10^3 cells/well in 8-chamber slides for ROS estimation and at a density of 3×10^4 cells/well in 6-well plates for RNA and protein extraction. Then, the cells were treated with 30.5 mM D-(+)-glucose (Sigma-Aldrich, St. Louis, MO, USA) with or without 5 μg/ml UCMA-FLAG protein for 24 or 72 hr, which were designated as high glucose and UCMA, respectively. The extraction and purification of UCMA-FLAG protein were performed by Thermo Fisher Scientific (Waltham, MA, USA) using the following method. Briefly, the expression vector containing the gene encoding UCMA-FLAG was prepared as previously described [14]. The vector was transfected into Expi293 cells, and the cells were lysed in a lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 0.1% Triton X-100, followed by sonication. The culture supernatants with the secreted UCMA-FLAG protein were centrifuged at 14,000 rpm for 1 min, and the UCMA-FLAG protein was purified through the FLAG-tag affinity column.

Determination of ROS production

MC3T3-E1 cells were treated with high glucose and UCMA for 24 hr and incubated in a medium containing 5 μM CellROX Green (Invitrogen, Waltham, MA, USA) for 30 min or 5 μM MitoSOX Red (Invitrogen) for 15 min in the dark. The cells were then mounted in an antifade solution containing 4',6-diamidino-2-phenylindole (DAPI; Invitrogen). CellROX- and MitoSOX-positive cells were detected at 485/520 and 510/580 nm, respectively, using a fluorescence microscope (Nuance FX). The fluorescence intensity was quantified using the ImageJ software (NIH, Bethesda, MD, USA).

Immunocytochemistry

After treating the MC3T3-E1 cells with high glucose and UCMA for 24 hr, they were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.01% Triton X-100 for 10 min, and blocked with 10% bovine serum albumin (Sigma-Aldrich) for 30 min at room temperature. After blocking, the cells were incubated overnight at 4℃ with an anti-heme oxygenase 1 (HO-1) antibody (Santa Cruz Biotechnology, Dallas, TX, USA), followed by incubation for 1 hr with Alexa Fluor 488-conjugated secondary antibody (Invitrogen). Then, the cells were mounted in an antifade solution containing DAPI to counterstain the nuclei. All images were obtained using a fluorescence microscope (Leica, Wetzlar, Germany).

RNA extraction and quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from the cells cultured for 72 hr using TRIzol reagent (Sigma-Aldrich), according to the manufacturer's instructions. cDNA was synthesized using a reverse transcriptase premix (ELPIS-Biotech, Daejeon, Korea) with 1 μg of total RNA, followed by qPCR using Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) on a StepOnePlus real-time PCR system (Applied Biosystems). The cycle progressed as follows: denaturation at 94℃ for 2 min; 40 cycles of amplification with denaturation at 94℃ for 30 s, annealing at 60℃ for 30 s, and extension at 72℃ for 30 s; and a final extension at 72℃ for 10 min. The following primers were used for qPCR: nuclear factor erythroid 2-related factor 2 (*Nrf2*, 5'-CTT AGA GGC TCA TCT CAC AC-3' and 5'-CAG CTT CCT TTT CCT ACA GT-3'), superoxide dismutase 1 (*SOD1*, 5'-CCA TCA GTA TGG GGA CAA TAC A-3' and 5'-GGT CTC

CAA CAT GCC TCT CT-3'), *SOD2* (5'-GAC CCA TTG CAA GGA ACA A-3' and 5'-GTA GTA AGC GTG CTC CCA CAC-3'), and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*, 5'-CAT CTC CCT CAC AAT TTC CA-3' and 5'-GTG CAG CGA ACT TTA TTG ATG G-3'). Results were analyzed using the comparative cycle threshold (C_T) method.

Protein extraction and western blotting

Total protein was extracted from the cultured cells using radioimmunoprecipitation assay buffer (Elpis Biotech) containing proteinase K (Roche, Basel, Switzerland). Equal amounts of protein were separated by 4%-20% mini-protein TGX precast protein gel (Bio-Rad, Hercules, CA, USA) electrophoresis and transferred onto PVDF membranes (Millipore, Burlington, MA, USA) according to standard immunoblot procedures. The membranes were incubated overnight at 4℃ with the following antibodies: anti-dynamin-related 1 (DRP1; Cell Signaling Technology, Danvers, MA, USA, 8570S, 1:1,000), anti-mitofusin-2 (MFN2; Cell Signaling Technology, 9482S, 1:1,000), anti-AKT (Cell Signaling Technology, #4685, 1:1,000), anti-phospho-AKT (p-AKT; Cell Signaling Technology, #4058, 1:1,000), and anti-β-ACTIN (Santa Cruz Biotechnology, sc-47778, 1:1,000). Bound antibodies were detected using an enhanced chemiluminescence kit (Bio-Rad). The intensities of protein bands were analyzed using the ImageJ software.

Statistical analysis

All data were analyzed using the Student's *t*-test and were presented as mean ± standard deviation (SD). A p value <0.05 was considered statistically significant.

Results

UCMA inhibited high glucose-induced ROS production in osteoblasts

Previous studies have demonstrated that UCMA treatment promotes the differentiation and nodule formation in MC3T3-E1 cells, suggesting its promising role in osteoblasts [14]. Another VKDP, OC, not only plays a critical role in calcification but also inhibits ROS production at high glucose levels [18, 24]. Based on this information, we investigated whether UCMA affects high glucose-induced ROS production in MC3T3-E1 cells treated with high concentrations of glucose, with or without UCMA. CellROX and MitoSOX staining were performed to determine cellular and mitochondrial ROS production, respectively. The results of CellROX and MitoSOX staining revealed increased ROS production in the cytoplasm and mitochondria of high glucose-treated MC3T3-E1 cells, respectively, which subsequently decreased upon the addition of UCMA (Fig. 1A). Moreover, quantification of CellROX- and MitoSOX-stained cells showed the increased intracellular ROS production in the cytoplasm and mitochondria of high glucose-treated cells, which decreased after subsequent treatment with UCMA (Fig. 1B).

To confirm the inhibition of ROS production by UCMA, the mRNA expression of antioxidant genes was measured using qPCR. The mRNA expression of *Nrf2* and *SOD1* tended

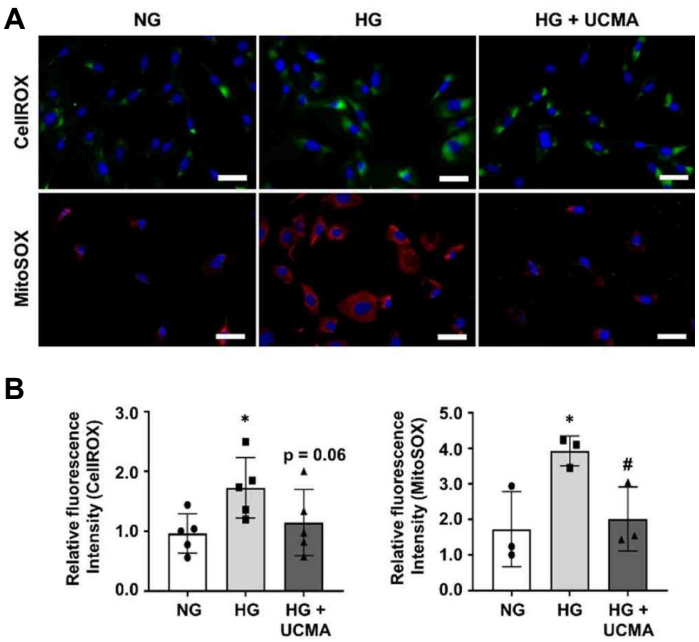

Fig. 1. ROS production in UCMA- and high glucose-treated osteoblasts. MC3T3-E1 cells were treated with NG, HG, or HG + UCMA for 24 hr. (A) CellROX and MitoSOX staining were performed to assess intracellular and mitochondrial membrane ROS production, respectively. Green and red indicate ROS stained by CellROX and MitoSOX, respectively; blue indicates the nucleus stained by DAPI. Scale bar, 5 μm. (B) The relative fluorescence intensity is plotted against the fluorescence intensity of NG-treated cells. Quantitative data are presented as mean ± SD. *p<0.05 compared to NG, #p<0.05 compared to HG. CellROX (n=5), MitoSOX (n=3). ROS, reactive oxygen species; UCMA, unique cartilage matrix-associated protein; NG, normal glucose; HG, high glucose; DAPI, 4',6-diamidino-2-phenylindole.

to decrease under high glucose conditions compared with that under normal glucose conditions in MC3T3-E1 cells, and the levels were significantly reversed by UCMA addition (Fig. 2A). *SOD2* expression, however, showed no significant differences among the three groups (Fig. 2A). Furthermore, exposure to high glucose concentrations resulted in HO-1 translocation from the cytoplasm to nucleus (Fig. 2B). Interestingly, UCMA treatment reduced the increase in nuclear HO-1 expression induced by high glucose concentrations (Fig. 2B). Collectively, these results suggested that UCMA ameliorated the high glucose-induced ROS production in osteoblasts.

UCMA increased mitochondrial fission in response to high glucose-induced ROS production

High glucose concentrations cause dysregulation of mitochondrial fission and fusion processes leading to ROS generation [36, 37]. DRP1 and MFN2 are involved in mitochondrial fission and fusion, respectively [10]. To investigate dysregulated mitochondrial fission and fusion, western blotting was performed using anti-DRP1 and anti-MFN2 antibodies. Although DRP1 expression did not differ between cells treated with high glucose alone and cells treated with normal glucose, its expression was increased in cells treated with both high glucose and UCMA compared with that in cells treated with high glucose alone (Fig. 3A). However, MFN2 expression showed no significant changes in any of the treatment groups (Fig. 3B). These results suggested that UCMA regulates mitochondrial fission by decreasing the high glucose-induced ROS production.

UCMA altered AKT signaling in response to high glucose-induced ROS production

AKT signaling is primarily involved in high glucose-induced ROS production [38]. To investigate the activation of AKT signaling, we performed western blotting using anti-AKT and anti-p-AKT antibodies to determine total AKT and activated AKT, respectively. The total expression of AKT showed no difference in any of the treatment groups. Consistent with DRP1 expression, the expression ratio of p-AKT/total AKT remained unchanged in cells treated with high glucose only compared with that in cells treated with normal glucose. However, the expression ratio of p-AKT/total AKT was significantly reduced in cells treated with both high glucose and UCMA compared with that in cells treated with

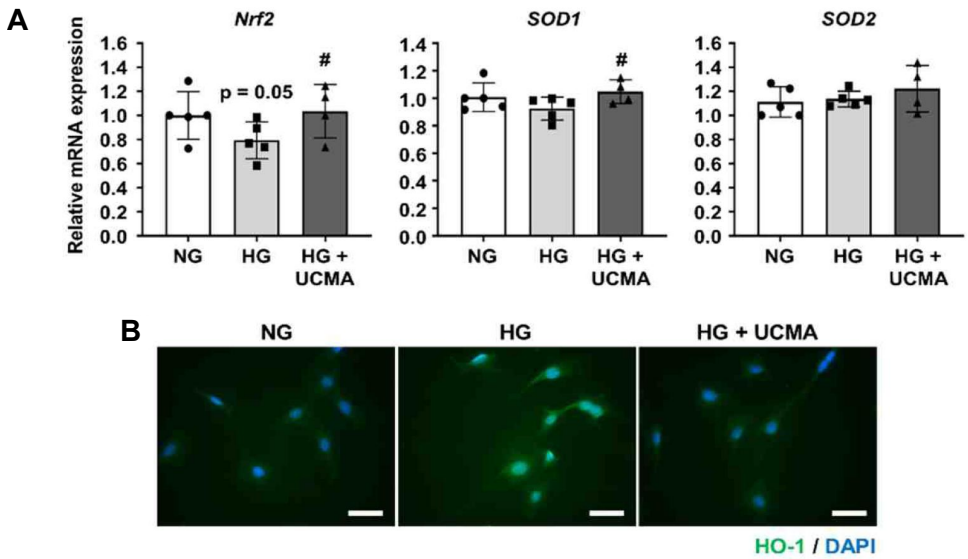

Fig. 2. Expression of antioxidant genes and HO-1 in UCMA- and high glucose-treated osteoblasts. (A) The mRNA expression of antioxidant genes was analyzed using qPCR. RNA was extracted from MC3T3-E1 cells treated with NG, HG, and HG + UCMA for 72 hr. The mRNA levels of *Nrf2*, *SOD1*, and *SOD2* were normalized to the expression level of *Gapdh*, and the relative mRNA expression levels were plotted against gene expression levels in NG-treated cells. Quantitative data are presented as mean ± SD. #p<0.05 compared to HG. NG (n=5), HG (n=5), HG + UCMA (n=4). (B) Immunocytochemistry was performed to detect the expression of HO-1. MC3T3-E1 cells were treated with NG, HG, and HG + UCMA for 24 hr. Green indicates HO-1 expression; blue indicates the nucleus stained by DAPI. Scale bar, 5 μm. UCMA, unique cartilage matrix-associated protein; NG, normal glucose; HG, high glucose; *Nrf2,* nuclear factor erythroid 2-related factor 2; *SOD*, superoxide dismutase; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; HO-1, heme oxygenase 1; DAPI, 4',6-diamidino-2-phenylindole.

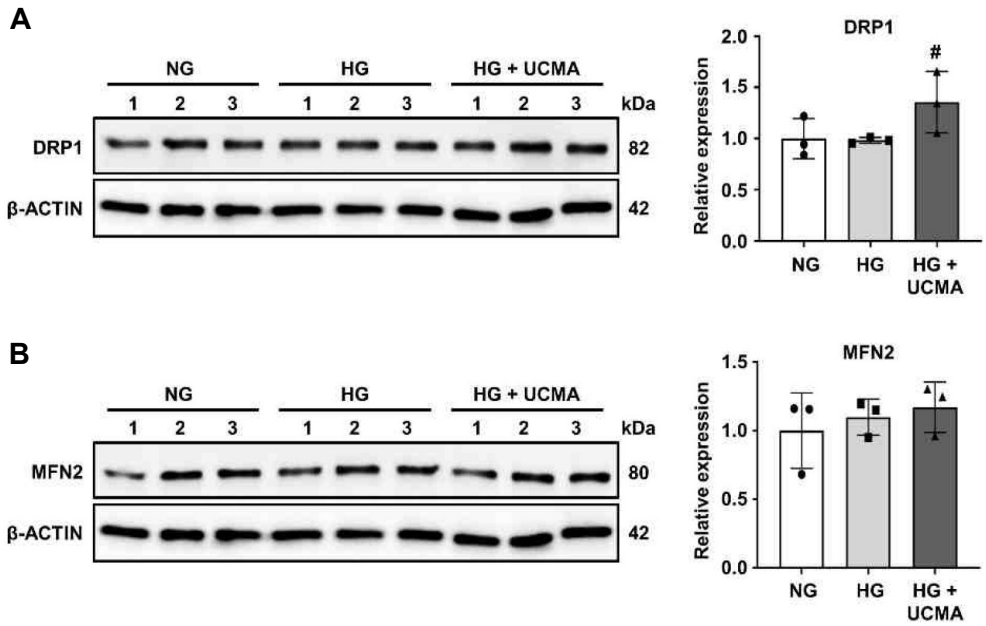

Fig. 3. Altered mitochondrial fission in UCMA-treated osteoblasts. Western blotting was performed to confirm the expression of proteins associated with mitochondrial fission using an anti-DRP1 antibody (A) and with mitochondrial fusion using an anti-MFN2 antibody (B). Proteins were extracted from MC3T3-E1 cells treated with NG, HG, and HG + UCMA for 72 hr. Quantitative analyses were performed, and the data are presented as mean ± SD. #p<0.05 compared to HG (n=3). Numbers represent experiments that were independently performed and collected in triplicate. UCMA, unique cartilage matrix-associated protein; NG, normal glucose; HG, high glucose; DRP1; dynamin-related protein 1; MFN2, mitofusin-2.

high glucose only (Fig. 4). These results suggested that UCMA inhibits AKT signaling by decreasing the high glucose-induced ROS production.

Discussion

UCMA is a secretory protein that belongs to the VKDP family and is primarily detected in cartilaginous tissues, bones, skin, and the vascular system [26, 30, 31]. Due to its Gla-rich composition, UCMA has a high binding affinity for calcium and thus plays a role in calcium deposition in bones [25]. Nonetheless, other functions of UCMA have also been demonstrated. Reportedly, UCMA inhibits cartilage degradation by regulating aggrecanase activity in inflammatory arthritis [23] and promotes osteoblast differentiation by directly binding to fibrillin-2 in osteoblasts [15]. It also exhibits anticancer properties by inhibiting the migration and invasion of triple-negative breast cancer cells [13]. Among VKDPs, OC ameliorates high glucose-induced ROS production through the AKT signaling pathway in osteoblasts [18]. Moreover, an increase in MGP expression is associated with reduced ROS levels in chondrocytes [6]. Our study demonstrated the role of UCMA in inhibiting ROS production in osteoblasts and in alleviating the impact of hyperglycemic stress. Osteoblasts treated with high glucose concentrations and UCMA displayed a drastic suppression of ROS production compared with that in osteoblasts treated with high glucose concentrations alone. Additionally, UCMA treatment increased the expression of *Nrf2* and *SOD1*, causing significant changes in mitochondrial fission, but not in fusion; therefore, it is necessary to determine the exact molecular mechanism of UCMA. Furthermore, UCMA treatment inhibited the activation of the AKT signaling pathway. It has been previously shown that the FLAG tag on the UCMA protein did not change the function of UCMA or have any cellular effect [14]. In addition, the concentration of UCMA used in this study was first selected based on cell viability assays, which demonstrated that 5 μg/ml of UCMA reduced high glucose-induced ROS production. Nevertheless, the dose or duration of UCMA treatment in this study could have been suboptimal, and further studies may be required.

HO-1 is an enzyme anchored in the endoplasmic reticulum and expressed in various intracellular compartments, including the mitochondria, nucleus, and plasma membrane [5]. Its translocation to the nucleus has been demonstrated in conditions associated with physiological stress, such as diabetes,

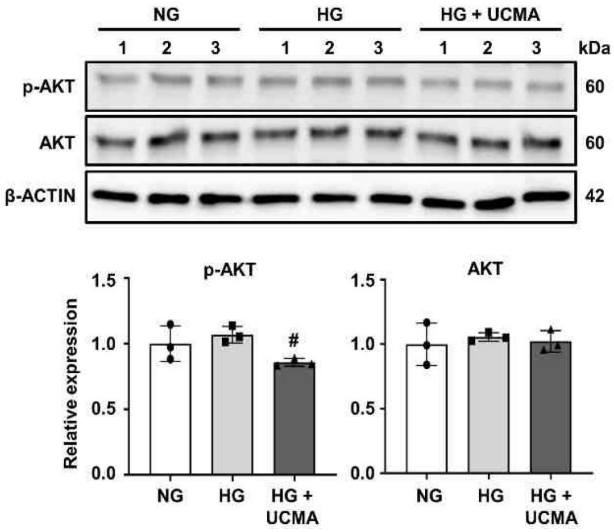

Fig. 4. Altered AKT signaling in UCMA-treated osteoblasts. Western blotting was performed to confirm the activation of AKT using anti-AKT and anti-p-AKT antibodies. Proteins were extracted from MC3T3-E1 cells treated with NG, HG, and HG + UCMA for 72 hr. Quantitative analyses were performed, and the data are presented as mean ± SD. #$p<0.05$ compared to HG (n=3). Numbers represent experiments that were independently performed and collected in triplicate. UCMA, unique cartilage matrix-associated protein; NG, normal glucose; HG, high glucose; p-AKT, phospho-AKT.

neurodegenerative diseases, and inflammatory diseases [22, 35]. *HO-1* is an oxidant-sensitive gene that is directly triggered by oxidative stress molecules, such as H_2O_2 and OH, and thiol-reactive substances, such as $CdCl_2$ and $NaAsO_2$ [21]. Moreover, HO-1 upregulation inhibits osteoblast mineralization and differentiation [16]. In this study, HO-1 was expressed in the nuclei of high glucose-treated osteoblasts and its nuclear expression was reduced in cells following UCMA addition. However, further investigations on the antioxidant effects of UCMA on osteoblasts treated directly with oxidative stress molecules or thiol-reactive substances may improve our knowledge of the other functions of UCMA.

Mitochondrial fission and fusion are essential for their normal function and determine their metabolic activity and ATP generation. Mitochondrial fission is proportional to the number of mitochondria in a cell and serves as a mechanism by which stress-damaged components are accumulated in daughter mitochondria and can be removed by autophagy [17, 27]. It also decreases membrane potential and causes autophagic removal of damaged cells [10]. Mitochondrial fusion minimizes mitochondrial dysfunction by decreasing the concentration of mutant mitochondrial genes and maintaining stable ATP production under stress conditions [40]. Excessive fission results in small mitochondrial fragments, whereas excessive fusion leads to the formation of elongated mitochondria. Mitochondrial fission and fusion are dynamically regulated by DRP1 and MFN2, respectively. DRP1-dependent fission is required in mitochondria during cell division and contributes to the elimination of damaged cells. Although alterations in mitochondrial fission were not directly observed in high glucose-treated osteoblasts with or without UCMA, DRP1 expression was increased in osteoblasts treated with high glucose levels and UCMA. However, the expression of proteins involved in mitochondrial dynamics under hyperglycemic conditions still remains controversial [12, 20], and further studies in this regard are warranted.

ROS activates the PI3K/AKT signaling pathway, which is essential for various cellular processes [39]. Hyperglycemia activates AKT signaling and induces MSC aging [38]. A previous study showed that OC reduces ROS production induced under hyperglycemic conditions through the AKT signaling pathway [18]. Our study showed the inhibitory activity of UCMA on the AKT signaling pathway activated by the high glucose-induced ROS production. Although we did not confirm the direct inhibitory role of UCMA by activation of AKT signaling under hyperglycemic conditions, a significant decrease in AKT signaling in cells treated with high glucose and UCMA suggests that UCMA is more effective in chronic hyperglycemia, although further research is required to determine this effect.

In conclusion, this study suggests a novel role for UCMA in elucidating the antioxidant effects of ameliorating hyperglycemic stress in osteoblasts.

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : Unique cartilage matrix-associated proteins에 의한 MC3T3-E1 조골세포에서의 고혈당 스트레스 완화 효과

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Unique cartilage matrix-associated protein (UCMA)은 γ-카르복실화(Gla) 잔기가 풍부한 간외 비타민 K 의존 단백질이다. UCMA는 조골세포 분화를 촉진하고 뼈 형성을 강화한다고 보고되고 있지만 고혈당 스트레스 하에서 조골세포에 미치는 영향에 대해서는 아직 알려진 바가 없다. 본 연구에서는 고혈당 조건하에서의 MC3T3-E1 조골세포에서 UCMA 효과를 조사하기 위해 MC3T3-E1 조골세포를 높은 포도당에 노출한 후 재조합 UCMA 단백질을 처리하였다. MC3T3-E1 세포에서 활성 산소종(ROS)의 생성은 고혈당 조건하에서 증가했으나 UCMA 단백질 처리 후 감소했음을 CellROX 및 MitoSOX 염색으로 확인하였다. 또한 고혈당 조건에서 UCMA 단백질을 함께 처리한 MC3T3-E1 세포에서 정량적 중합효소 연쇄반응 결과, 항산화 유전자인 nuclear factor erythroid 2-related factor 2 와 superoxide dismutase 1 발현이 증가하였다. 동일 조건하에서 UCMA 단백질 처리에 의해 heme oxygenase-1 발현 감소와 함께 세포질에서 핵으로의 전위가 감소되었고, 미토콘드리아 분열에 관여하는 dynamin-related protein 1 발현이 증가하였으며, AKT 신호 활성은 억제되었다. 종합적으로 UCMA는 고혈당에 노출된 조골세포에서 ROS 생성을 완화하고, 항산화 유전자 발현을 증가시키고, 미토콘드리아 역학에 영향을 미치며, AKT 신호를 조절하는 것으로 보인다. 본 연구는 UCMA의 세포 메커니즘에 대한 이해를 돕고, 대사 장애 관련한 골 합병증에 대한 새로운 치료제로서의 잠재적 사용 가능성을 제시하고 있다.