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7-Ketocholesterol accelerates pancreatic β-cell senescence by inhibiting the SIRT1/CDK4–Rb–E2F1 signaling pathway

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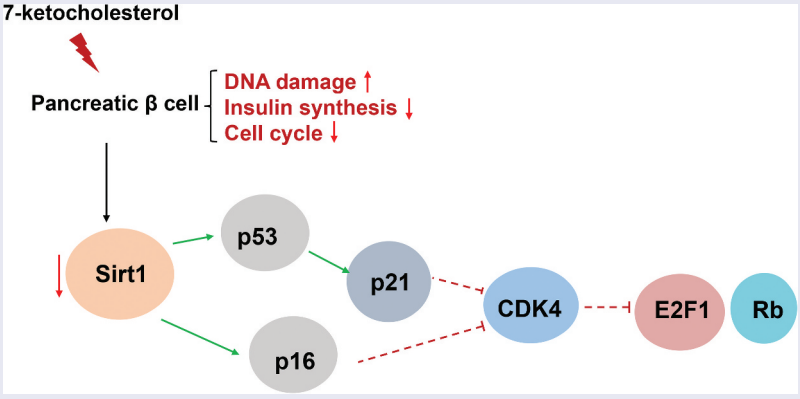
ABSTRACT

Pancreatic β-cell dysfunction is a key factor in the development of type 2 diabetes. Pancreatic β-cell senescence accelerates abnormal glucose metabolism, which decreases insulin secretion and cell regeneration ability, eventually leading to diabetes. A cholesterol oxidation product, 7-ketocholesterol (7-KC) can affect pancreatic β-cell function. However, its role in pancreatic β-cell senescence has not been reported. We investigated the role of 7-KC in pancreatic β-cell senescence and its underlying molecular mechanism in MIN6 cells. MIN6 cells were treated with 25 μmol/L 7-KC for 24 h and the proportion of senescent cells was detected based on senescence-associated β-galactosidase (SA-β-gal) activity. The cell cycle, DNA damage, and the senescence-associate secretory phenotype (SASP) and protein expression were detected by flow cytometry, immunofluorescence, and western blotting, respectively. 7-KC can significantly increase SA-β-gal activity, promoted G0/G1 arrest, DNA damage, and interleukin-1β expression in MIN6 cells and significantly inhibited insulin synthesis. Further studies indicated that 7-KC induced β-cell senescence by inhibiting the SIRT1/CDK4–Rb – E2F1 signaling pathway.



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Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disease characterized by insulin resistance and pancreatic β-cell dysfunction. Obesity and abnormal glucose and lipid metabolism can cause pancreatic β-cell senescence and lead to β-cell dysfunction.[1] Increased numbers of senescent pancreatic β-cells accelerate the gradual deterioration of β-cell function and cell secretion dysfunction.[2] Moreover, senescence-associated secretory phenotype (SASP) secreted by senescent cells can trigger chronic, low-level, systemic inflammation that accelerates the pancreatic β-cell senescence process.[3]

Pancreatic β-cell lipotoxicity and glucolipotoxicity may contribute to pancreatic β-cell senescence. Previously, we demonstrated that cholesterol metabolism disorder inhibited glucose-stimulated pancreatic β-cell insulin secretion.[4,5] Excess cholesterol reduced glucose transporter activity to influence glucose uptake, reduce glucokinase activity, and inhibit glycolysis.[6–9] Cholesterol is converted to other

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products via oxidation/reduction *in vivo*, where 7-ketocholesterol (7-KC) is the main oxidation product following auto-oxidation. 7-KC demonstrates extensive toxicity and is difficult to metabolize for most cells. Consequently, 7-KC accumulation in organisms can damage cell and tissue functional activity[10–13] and is directly related to many diseases, including metabolic disease, atherosclerosis, heart failure, age-related macular degeneration, and Alzheimer disease.[14,15] Non-alcoholic fatty liver disease is caused by hepatocyte ballooning, inflammation, and steatosis due to the accumulation of oxysterols, specifically 7-KC.[16,17] 7-KC is toxic to macrophages, where it promotes inflammation via the LXR and hepatocyte nuclear factor 4 (HNF-4) signaling pathway in atherosclerotic plaques[16,18] 7-KC induces peroxisomal disorders in glial, microglial, and neuronal cells and induces mitochondrial dysfunctions, oxidative stress, and inflammation, which promote neurod egeneration.[19,20] However, the role of 7-KC in pancreatic β-cell senescence has not been reported.

Based on the above reports, we examined whether 7-KC affects pancreatic β-cell senescence and elucidated the mechanism of β-cell senescence. Our results indicated that 7-KC accelerated pancreatic β-cell senescence, which occurred via SIRT1/CDK4–Rb – E2F1 signaling pathway inhibition.

Materials and methods

Reagents and antibodies

The hydrogen peroxide (H_2O_2) was from Sigma-Aldrich (St. Louis, MO, USA). The 7-KC was from Sigma-Aldrich and dissolved in 45% (w/v) 2-hydroxypropyl-β-cyclodextrin solution (Sigma-Aldrich). Type-V collagenase (C9263) was fromSigma-Aldrich (St. Louis, MO, USA). SA-β-gal activity kit was from GenMed Scientific (Shanghai, China). Cell cycle and apoptosis analysis kit was from Beyotime (Shanghai, China). The antibodies against CDKN2A/p16INK4a (ab211542), γ-H2A.X (ab81299), p21 (ab88224), SIRT1 (ab189494), CDK4 (ab68266), and phosphorylated retinoblastoma (p-Rb) protein (ab184796) were from Abcam (Cambridge, UK). The antibodies against E2F1 (A19579), insulin (a19066) were from ABclonal (Wuhan, China). The anti-p53 antibody (10442–1-AP) was from Proteintech (Wuhan, China). The anti-p-p38MAPK antibody (4511) was from Cell Signaling Technology (Boston, MA, USA). The anti-β-gal antibody (A11132) was from Thermo Fisher Scientific (Waltham, MA, USA).

Cell culture

The 35th to 38th generation MIN6 cells were cultured in high-glucose Dulbecco's modified Eagle's medium containing 15% fetal bovine serum, 1% penicillin/streptomycin, and 0.5‰ β-mercaptoethanol at 37°C in a humidified atmosphere under 5% CO_2 for 16 h. Then, the cells were exposed to 25 μmol/L 7-KC according to our previous study before they were harvested. The positive control was 150 μmol/L H_2O_2.[21]

Islet isolation

The mice were anesthetized and immediately process for pancreatic perfusion with 3 mL fresh type-V collagenase dissolved in HBSS (Hank's Buffered Salt Solution). The perfused pancreas was quickly incubated at 37°C for 28 min, and terminated by adding HBSS buffer containing 10% FBS, then the islets were purified by density gradient centrifugation. The islets were picked by hand under an anatomical microscope. After washing with HBSS, the islets were cultured in RPMI-1640 medium with 10% FBS overnight. Then, the islets were exposed to different concentrations 7-KC for 24 h.

SA-β-gal assay

MIN6 cells were seeded on 6-well plates and incubated as described in the Cell Culture section. After 24-h incubation in the fixative solution provided in the commercial kit, cell senescence was assessed by X-gal staining for detecting β-gal activity. Images were recorded with an optical microscope (Carl Zeiss, USA), where stained cells in 10 different fields were counted and the percentage of positive cells was calculated by Image J software.

Cell cycle determination

MIN6 cells were seeded on 6-well plates and incubated as described in the Cell Culture section. Subsequently, cycle analysis was performed as per the manufacturer's instructions using cell cycle and apoptosis detection reagents. The percentage of apoptotic cells was quantified by measuring G1 cell levels using flow cytometry (BD bioscience, USA) and ModFit LT software.

Immunofluorescence assay

MIN6 cells were plated on cell slides in 6-well plates and incubated as described in the Cell Culture section. Then, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100/phosphate-buffered saline (PBS) and blocked with 5% bovine serum albumin (BSA) for 60 min. The cells on coverslips were incubated overnight at 4°C with the primary antibodies. After several washes in PBS, the samples were incubated with the fluorescent secondary antibody (1:200 dilution; Invitrogen, Carlsbad, CA, USA) at room temperature for 60 min. After rinsing with PBS, the samples were stained for 15–30 min with 4′, 6-diamidino-2-phenylindole (DAPI) working solution. After mounting with fluorescence decay-resistant medium, the cells were observed and photographed under a confocal microscope (IX83-FV3000, Olympus, Tokyo, Japan).

Western blotting

The MIN6 cells or islets were lysed on ice with cell lysis buffer and the protein concentration was determined by a bicinchoninic acid kit (FdBio Science, Hangzhou, China). Then, the sample underwent 10% or 15% sodium dodecyl sulfate – polyacrylamide gel electrophoresis and were transferred to polyvinylidene fluoride membranes. After incubation with BSA, primary antibodies were added to the sample and incubated at 4°C overnight. Subsequently, the sample was incubated for 2 h with horseradish peroxidase-conjugated secondary antibodies at room temperature. Then, the protein was detected by a chemiluminescence ECL kit (FdBio Science) and the protein bands were analyzed by ImageJ.

Statistical analysis

The experimental data are expressed as the mean ± SEM. The data were statistically analyzed with SPSS 20 and plotted with GraphPad Prism 5. The experiment in each group was repeated at least three times independently, and the statistical significance of the difference between the experimental and control groups was determined by an independent sample t-test. $P < 0.05$ was set as the threshold for statistical significance.

Results

7-KC significantly induced senescence in pancreatic β cell

As β-gal is a characterized senescence marker, we detected its activity in 7-KC-treated MIN6 cells. Aguayo-Mazzucato et al. demonstrated that H_2O_2 induced β-cell senescence and led to the loss of β-cell identity[22] . Accordingly, we used H_2O_2 as the positive control in our study. In this study, the positive rate of SA-β-gal in the normal control (NC), 7-KC, and H_2O_2 groups was 8.1% ± 1.4%, 98.7% ± 4.6%, and 58.6% ± 3.6%, respectively (Figures 1A, B). Compared with the NC group, the 7-KC and H_2O_2 groups exhibited obviously enhanced SA-β-gal staining ($P < 0.01$), where the 7-KC group had more significantly enhanced staining than the H_2O_2 group ($P < 0.05$). Moreover, we detected the β-gal expression in islets. The results showed the β-gal expression was increased in 25 and 50 μmol/L 7-KC treatment group (Figures 1G, H).

The p53–p21 and $p16^{INK4a}$ – Rb axes establish and maintain cell senescence.[23] The p53–p21 axis initiates the aging process while $p16^{INK4a}$ activation maintains the aging state.[22] Senescent cells contain increased levels of the senescence-related proteins p16, p53, and p21.[24] To study the effect of 7-KC on pancreatic β-cell senescence, we detected the expression of senescence-related proteins in each group with western blotting. 7-KC significantly upregulated p53, p21, and p16 expression in the

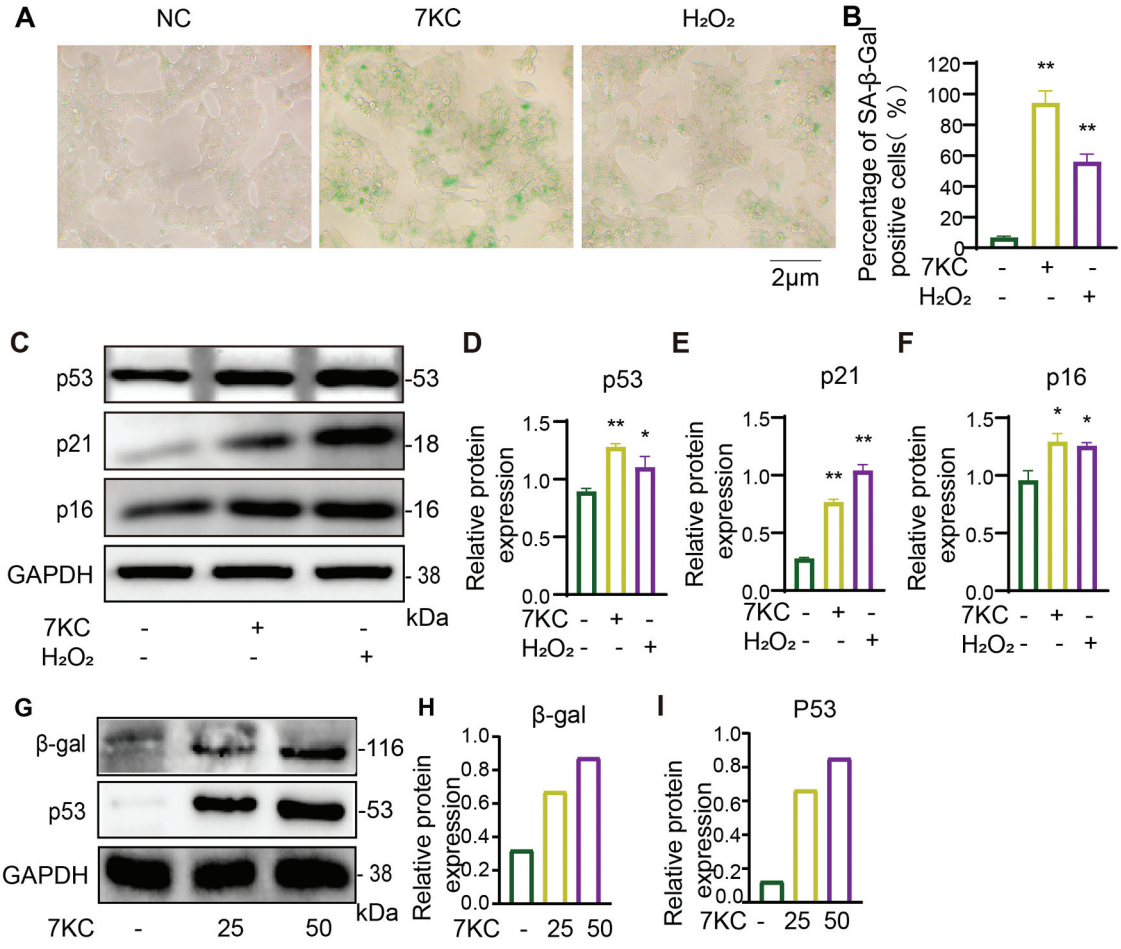

Figure 1. 7-KC significantly induced senescence in pancreatic β cell. (A, B) SA-β-gal activity detection after 7-KC treatment in MIN6 cell. (C – F) p53, p21, and p16 expression after 7-KC or H_2O_2 treatment in MIN6 cell. (G – I) β-gal and p53 expression after 25 and 50 μmol/L 7-KC treatment in islets. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. Normal control. The senescence staining, senescence markers p53, p21 and p16 were detected by SA-gal staining and Western blot after 7-KC or H_2O_2 treatment in pancreatic β cell for 24 h. **(A, B)** SA-β-gal activity detection after 7-KC or H_2O_2 treatment in MIN6 cell for 24 h. **(C – F)** p53, p21, and p16 expression after 7-KC or H_2O_2 treatment in MIN6 cell for 24 h. Normal control (2-hydroxypropyl-β-cyclodextrin solution); 7-KC (25 μmol/L 7-KC in 2-hydroxypropyl-β-cyclodextrin solution); H_2O_2(150 μmol/L H_2O_2+2-hydroxypropyl-β-cyclodextrin solution). (**G – I**) β-gal and p53 expression after 25 and 50 μmol/L 7-KC treatment in islets for 24 h. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. Normal control.

pancreatic MIN6 cells as compared with that in the NC group (Figure 1C-F). And the p53 expression in islets was increased at 25 and 50 μmol/L 7-KC treatment group, the result accorded with in MIN6 cell (Figures 1G, I).

7-KC significantly arrested the cell cycle of MIN6 cells

Cellular senescence is a terminal state of growth arrest where cells cannot reenter the cell cycle despite mitogenic growth signals, which is termed cell cycle arrest.[25] To verify the effect of 7-KC on the cell cycle, we detected the proportion of G1-, G2-, and S-phase MIN6 cells by flow cytometry. Compared with the NC, 7-KC significantly increased G0/G1 cells and decreased the proportion of S-phase cells. The proportion of G1 MIN6 cells was increased in both the 7-KC and H_2O_2 groups as compared with those in the NC group, but there was no significant difference between the 7-KC and H_2O_2 groups (Figures 2A, B). These findings suggested that 7-KC inhibited cell proliferation by inhibiting the cell cycle.

7-KC significantly increased the phosphorylation of histone variant H2A.X (*γ-H2A.X*) in MIN6 cells

Cell senescence can also be characterized by increased the phosphorylation of histone variant

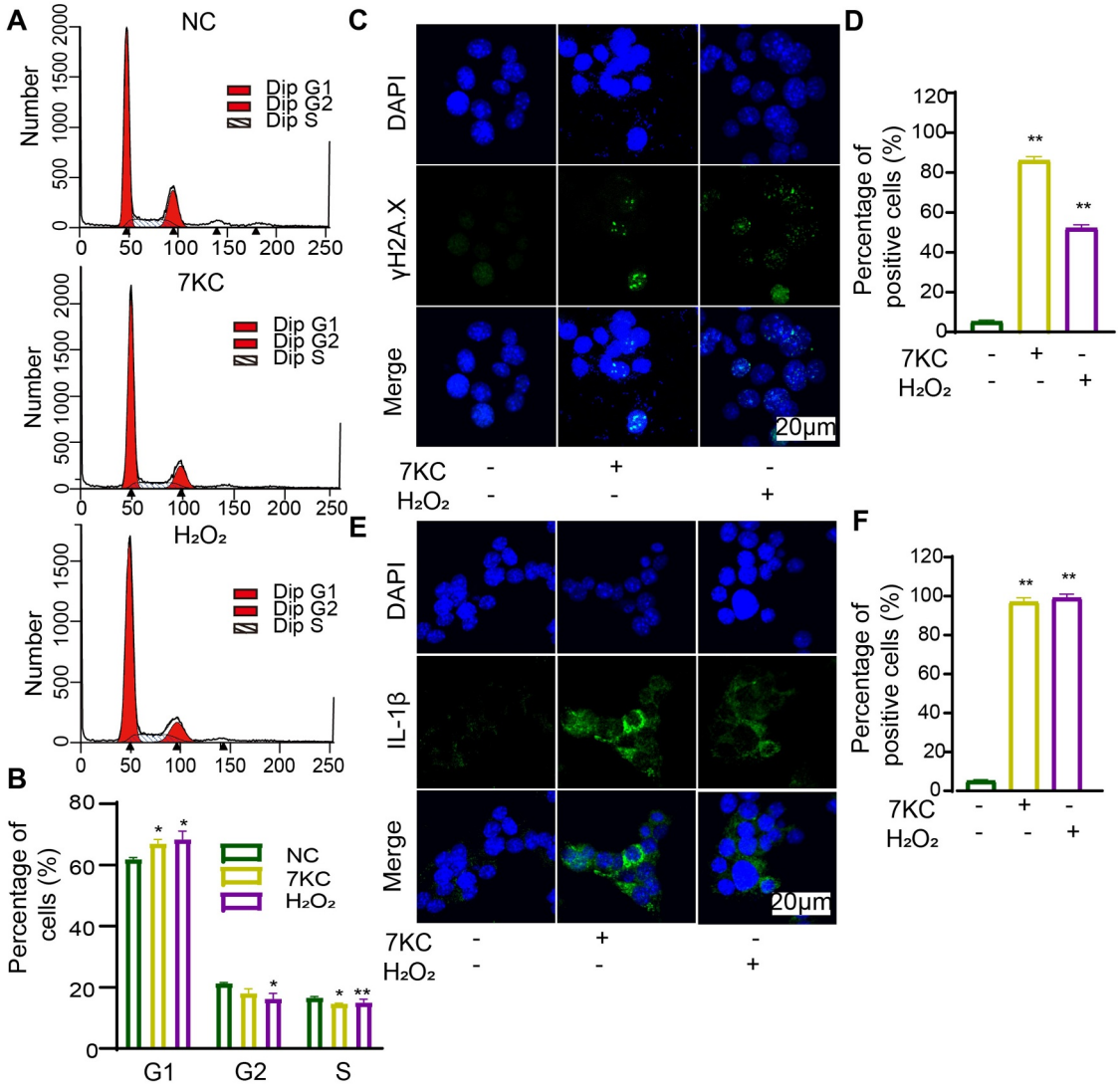

Figure 2. 7-KC or H_2O_2 significantly promoted the senescence phenotype in MIN6 cells. (A, B) Flow cytometry cell cycle analysis after 7-KC or H_2O_2 treatment. (C, D) Immunofluorescence detection of γ-H2A.X expression. (E, F) Immunofluorescence detection of IL-1β expression. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. Normal control. The cell cycle was inhibited, γ-H2A.X and IL-1β expression were increased after 7-KC or H_2O_2 treatment. **(A, B)** Flow cytometry cell cycle analysis after 7-KC or H_2O_2 treatment. **(C, D)** Immunofluorescence detection of γ-H2A.X expression. **(E, F)** Immunofluorescence detection of IL-1β expression. Normal control (2-hydroxypropyl-β-cyclodextrin solution); 7-KC (25 μmol/L 7-KC in 2-hydroxypropyl-β-cyclodextrin solution); H_2O_2(150 μmol/L H_2O_2+2-hydroxypropyl-β-cyclodextrin solution). $^{**}P < 0.01$, $^{*}P < 0.05$ vs. Normal control.

H2A.X (γ-H2A.X).[26] The γ-H2A.X level can clearly reflect the degree of DNA damage and DNA repair. (Figures 2C , 2D) depict the γ-H2A.X expression level detected by immunofluorescence staining. The proportion of γ-H2A.X-positive cells in the NC, 7-KC, and H_2O_2 groups was 5.2% ± 1.2%, 86% ± 3.4%, and 52% ± 3.1%, respectively. Our findings demonstrated that 7-KC exposure significantly promoted DNA double-strand break in pancreatic MIN6 cells.

7-KC significantly promoted inflammatory cytokine expression in MIN6 cells

The SASP accompanies the permanent cell cycle arrest of senescent cells and consists of proinflammatory factors such as cytokines, chemokines, and extracellular matrix remodeling

factors. The proinflammatory cytokines consist of interleukin IL-1α, IL-1β, IL-6, and IL-8.[5] The above results demonstrated that 7-KC arrested the cell cycle of β-cells. Subsequently, we studied the effect of 7-KC on inflammatory cytokine expressed from MIN6 cells, where the proportion of IL-1β-positive cells was detected by immunofluorescence. The percentages of IL-1β-positive cells among the NC-, 7-KC-, and H_2O_2-treated MIN6 cells were 4.7% ± 1.1%, 97% ± 3.8%, and 99% ± 3.5%, respectively (Figures 2E, F). The results demonstrated that 7-KC significantly promoted IL-1β expression in the pancreatic β-cells.

7-KC significantly inhibited MIN6 cell insulin expression

The earlier results demonstrated that 7-KC caused β-cell senescence and attenuated β-cell insulin secretion (unpublished data). To confirm the effect of 7-KC on insulin secretion, we detected β-cell insulin expression with immunofluorescence. The positive rates of insulin expression in the NC, 7-KC, and H_2O_2 groups were 99.2% ± 4.2%, 67% ± 3.7%, and 65% ± 3.4%, respectively (Figure 3). Compared with the NC, 7-KC significantly down-regulated insulin expression, but there was no significant difference between the effects of 7-KC and that of H_2O_2.

7-KC caused MIN6 cell senescence possibly through the SIRT1–CDK4–Rb – E2F1 signaling pathway

SIRT1 plays a major role in metabolic disease attenuation, lifespan extension, antioxidation, and cellular senescence inhibition.[27] SIRT1 activation protected pancreatic β-cells against palmitate-induced dysfunction.[28] Zhang et al. reported that SIRT1 deficiency impaired normal cell cycle progression and that cyclin B1 and p-CHK2 expression was increased in $SIRT1^{-/-}$ brains.[29] Many CDK and cyclin complexes control the cell cycle. The cyclin D – CDK4/6 complex phosphorylates the Rb C-terminal to prevent active transcription. When stimulated by mitosis, cyclin D – CDK4/6 initiates the phosphorylation of pocket proteins, leading to the destruction of the E2F/pocket

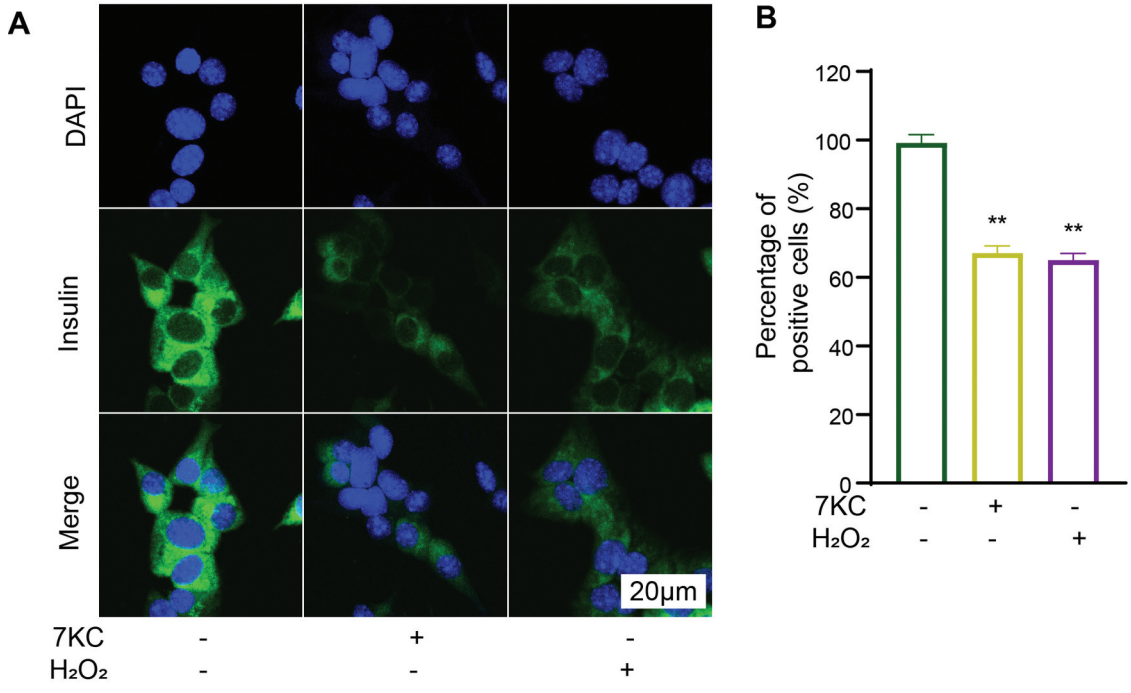

Figure 3. 7-KC or H_2O_2 significantly inhibited insulin expression in MIN6 cells. (A, B) Immunofluorescence detection of insulin expression. $^{**}P < 0.01$ vs. Normal control. The immunofluorescence staining showed the 7-KC or H_2O_2 significantly inhibited insulin expression in MIN6 cells. **(A, B)** Immunofluorescence detection of insulin expression. Normal control (2-hydroxypropyl-β-cyclodextrin solution); 7-KC (25 μmol/L 7-KC in 2-hydroxypropyl-β-cyclodextrin solution); H_2O_2(150 μmol/L H_2O_2+2-hydroxypropyl-β-cyclodextrin solution). $^{**}P < 0.01$ vs. Normal control.

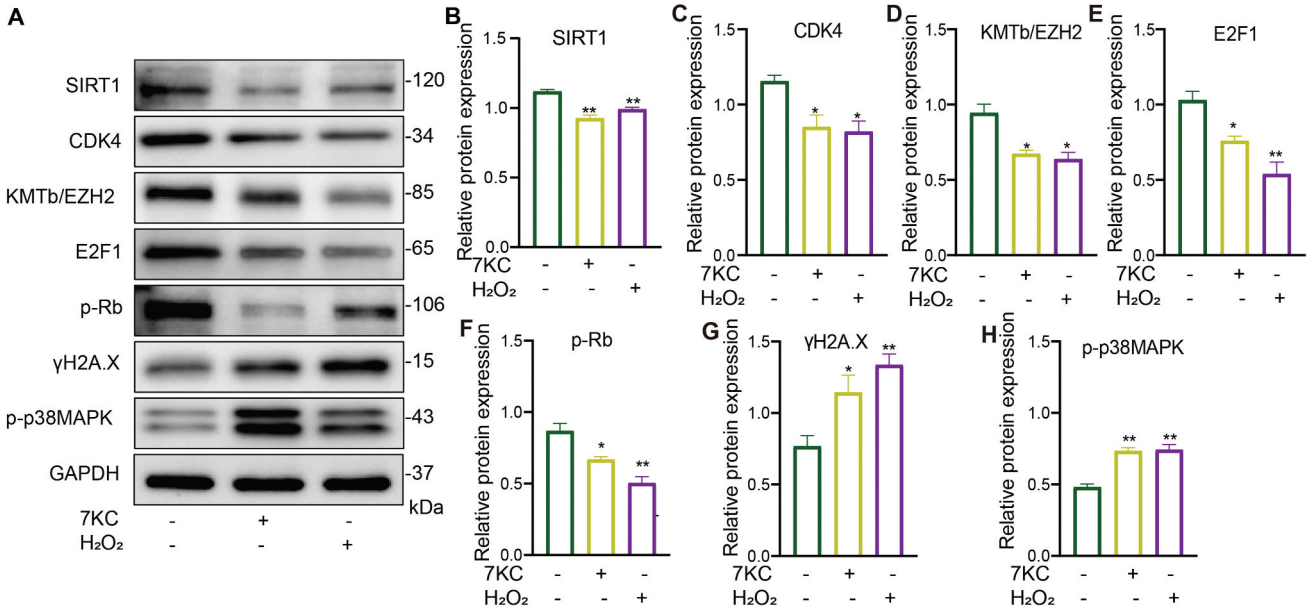

Figure 4. 7-KC or H_2O_2 caused MIN6 cell senescence through the SIRT1–CDK4–Rb – E2F1 signaling pathway. (A – H) MIN6 cells were treated with 7-KC or H_2O_2 for 24 h, then SIRT1 (B), CDK4 (C), KMTb/EZH2 (D), E2F1 (E), *p*-Rb (F), γ-H2A.X (G), and p-p38MAPK (H) expression was detected by western blotting (A). *P < 0.05, **P < 0.01 vs. Normal control. The protein expression showed that 7-KC or H_2O_2 caused MIN6 cell senescence through the inhibiting SIRT1 and CDK4–Rb – E2F1 signaling pathway. **(A – H)** MIN6 cells were treated with 7-KC or H_2O_2 for 24 h, then SIRT1 (**B**), CDK4 (**C**), KMTb/EZH2 (**D**), E2F1 (**E**), p-Rb (**F**), γ-H2A.X (**G**), and p-p38MAPK (**H**) expression was detected by western blotting (**A**). Normal control (2-hydroxypropyl-β-cyclodextrin solution); 7-KC (25 μmol/L 7-KC in 2-hydroxypropyl-β-cyclodextrin solution); H_2O_2(150 μmol/L H_2O_2 in 2-hydroxypropyl-β-cyclodextrin solution). *$P < 0.05$, **$P < 0.01$ vs. Normal control.

protein inhibitory complex and the nuclear output of E2F.[30] Figure 4 demonstrates that 7-KC significantly inhibited SIRT1 expression, which agreed with the β-gal staining results. Moreover, CDK4, EZH2, and E2F1 expression and Rb phosphorylation were inhibited while γ-H2A.X expression and p38MAPK phosphorylation (Figure 4) were upregulated in the MIN6 cells. These results suggested that 7-KC regulated the MIN6 cell senescence process through the SIRT1–CDK4–Rb – E2F1 signal pathway.

Discussion

The loss of functional β-cells is the key mechanism leading to diabetes. Senescent β-cells accumulate during obesity and aging. Pancreatic β-cell senescence was observed in a high-fat-fed mouse model and was followed by insufficient insulin release.[31] Using oral ABT263 to remove senescent β-cells improved glucose metabolism and β-cell function in high-fat diet C57BL/6 mice.[22] These studies suggested that lipotoxicity caused the β-cell senescence. Previous studies also demonstrated that cholesterol accumulation damaged β-cell function. Cholesterol accumulation affects the metabolic process of glucose-stimulated insulin release. Patients with T2DM present a cluster of lipid abnormalities associated with cholesterol and fatty acid accumulation in pancreatic β-cells, which may contribute to islet degeneration.[6] 7-KC is the main cholesterol auto-oxidation product, where accumulated 7-KC can exert various adverse effects and is associated with many diseases. Our results demonstrated that 7-KC clearly increased the proportion of β-gal cells and the senescence markers p16, p21, and p53 were increased in the 7-KC group. Many studies demonstrated that H_2O_2 is the classical model for inducing cell senescence[22,32,33] and β-gal staining and protein expression confirmed that H_2O_2 induced cell senescence. Compared with H_2O_2, 7-KC accelerated β-cell senescence in an obvious manner. The results suggested that 7-KC affects β-cell function and may accelerates the T2DM process.

Senescent cells are characterized by increased cell size and lysosome content, which upregulate β-gal activity and DNA damage, inhibiting cell

proliferation, arresting the cell cycle, and upregulating CDKN2A/1A (encoding p16INK4a and p21, respectively), anti-apoptotic molecules (Bcl-2, Bcl-xL, Bcl-w), and SASP factors.[22,34–36] The DNA damage level increases with senescence while DNA repair ability decreases, rendering β-cells more prone to senescence-related cell cycle arrest and the DNA damage response.[37] Multiple proteins regulate the cell cycle. p16 is a cyclin kinase inhibitor that participates in cell cycle regulation and competes with cyclin D1 (cyclin D1) to bind CDK4 and CDK6, specifically inhibiting CDK4 activity, preventing S phase entry and DNA synthesis initiation, and inhibiting Rb protein phosphorylation.[38] Unphosphorylated Rb binds to E2F1 to inhibit the activation of E2F1 and cell proliferation.[39,40] EZH2 downregulation in aging pancreatic β-cells leads to the activation of p16INK4a and p19ARF sites and decreased pancreatic β-cell function.[41] Our results demonstrated that 7-KC treatment of MIN6 cells promoted G0/G1 arrest, which was regulated by inhibiting CDK4, E2F1, and EZH2, and Rb phosphorylation. Furthermore, the phosphorylation level of the histone γ-H2A.X, a DNA damage marker, was increased in an obvious manner. Senescent cells trigger profound phenotypic changes, such as the production of a bioactive secretome, referred to as the SASP.[42] Our results demonstrated that 7-KC treatment increased IL-1β levels. The results are in accordance with reports that IL-1β was increased in β-cells and was an independent predictor of diabetes.[21,43,44]

SIRT1 is strongly implicated in obesity and diabetes. SIRT1 decreases the inflammatory process by affecting the NF-κB pathway. In *Sirt1* knockout mice, high-fat diet lead to upregulated insulin resistance.[45] SIRT1 activation promoted β-cell regeneration by activating AMPK signaling-mediated fatty acid oxidation.[46] The islets from *Sirt1* knockout mice exhibited reduced glucose-stimulated insulin secretion.[47] In rats, resveratrol attenuated β-cell senescence induced by excessive ethanol exposure via the SIRT1–p38MAPK – p16 pathway.[27] Our results are consistent with the previous studies in which *Sirt1* was obviously decreased after 7-KC treatment.[45,46]

In summary, our experimental data demonstrated that DNA damage induced p21, p16, and p53 expression, which was accompanied by decreased DNA repair (SIRT1 downregulation), CDK4 inhibition, decreased Rb phosphorylation, and the limitation of E2F1 activation. These changes promoted G1 arrest in MIN6 β-cells. In this study, we not only elucidated the potential mechanism of 7-KC in pancreatic β-cell senescence, but may also suggested potential therapeutic targets for preventing and treating pancreatic β-cell senescence-related diseases such as T2DM.

Our study indicated that 7-KC led to pancreatic β-cell senescence possibly by regulating SIRT1/CDK4–Rb – E2F1 signal pathway activation.

Acknowledgments

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Disclosure statement

No potential conflict of interest was reported by the authors.

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