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Hyperglycemic stress induces oxidative damage of enteric glial cells by triggering redoxosomes/p66SHC activation

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ABSTRACT

Objectives: Diabetic gastrointestinal dysfunction (DGD) is a serious complication of diabetic mellitus (DM), affecting the enteric nervous system (ENS), particular enteric glial cells (EGCs). This study aimed to elucidate the effects and underlying molecular mechanisms of hyperglycemic stress on EGCs in *in vitro* and *in vivo* models of DM.

Methods: In *in vitro* studies, enteric glial cell line CRL-2690 was exposed to hyperglycemia stress, and cell viability, cell apoptosis and oxidative damage were assessed. In *in vivo* studies, STZ-induced diabetic mice were constructed, and cell apoptosis and oxidative damage of EGCs in the duodenum of DM mice were assessed.

Results: The results showed that hyperglycemic stress markedly induced oxidative damage of EGCs in *in vitro* and *in vivo* models of DM. This damage was found to be dependent on the activation of redoxosomes, which involved the phosphorylation of SRC and Vav2, the up-regulation of active RAC1-GTP, and the activation of NADPH oxidase (NOX). Moreover, inhibitors of redoxosomes, such as the RAC1 inhibitor NSC23766 and the NOX inhibitor VAS2870, effectively mitigated the hyperglycemic stress-induced oxidative damage of EGCs. Additionally, inhibition of p66SHC, a downstream target of redoxosomes, attenuated oxidative damage of EGCs under hyperglycemic stress.

Discussion: Our findings suggest that the redoxosomes/p66SHC signaling is involved in the oxidative damage of EGCs during the pathological process of DGD. This signaling cascade may represent a potential therapeutic target for the treatment of DGD.

KEYWORDS
Diabetic gastrointestinal dysfunction; enteric glial cells; hyperglycemic stress; redoxosomes/p66SHC signaling

1. Introduction

Diabetic gastrointestinal dysfunction (DGD), a common complication of diabetic mellitus (DM), often occurs in patients with longstanding diabetes and poorly controlled hyperglycemia [1,2]. The typical symptoms of DGD include heartburn, abdominal pain, nausea, vomiting, constipation, diarrhea, and fecal incontinence, severely impacting on life quality of diabetic patients [3]. The enteric nervous system (ENS) is the largest division of the peripheral nervous system in guts, and the essential role of the ENS in DGD is well studied [4,5]. Enteric glial cells (EGCs) are an integral part of ENS in the gastrointestinal tract. They are involved in neuroprotection, immune system modulation and many other physiological processes [6,7]. Recently, EGCs have been proposed to be a crucial target in the control of DGD [8]. Our previous study has reported that circVPS13A overexpression attenuates diabetes-induced damages of EGCs in both *in vitro* and *in vivo* DM models [9]. Another study has demonstrated that EGCs activation protects enteric neurons from diabetic toxicity via promoting neurotrophic factor release [10]. However, the impact of hyperglycemia stress on EGCs oxidative injury during the pathogenesis of DGD still remain unclear.

Oxidative stress refers to a distortion in the redox balance of cells under pathological condition, leading to the damage of DNA, proteins and lipids [11,12]. Oxidative stress has recently been recognized as a key player in the pathogenesis of DGD [13]. Increased oxidative stress is associated with a loss of inhibitory neuronal subpopulation of enteric neurons in DGD patients [13]. Redoxosomes are redox-active endosomes and can transmit ROS (reactive oxygen species) signals from the endosome interior to redox-sensitive effectors on the endosomal surface [14]. Hyperglycemic stress in the kidney or retina is thought to be the trigger of redoxosomes activation, resulting in oxidative damage and cell deaths [15–17].

In the present study, we used CRL-2690 cells exposed to hyperglycemia as *in vitro* model and STZ (streptozotocin)-induced diabetic mice as *in vivo* model to explore the role of redoxosomes/p66SHC signaling in hyperglycemic stress-induced oxidative damage in EGCs of DGD.

2. Materials and methods

2.1 Chemicals and reagents

Chemicals such as RAC1 inhibitor NSC23766 and NOX (NADPH oxidase) inhibitor VAS2870 were purchased from MCE Biotechnology. MTT (3-(4,5-Dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide) cell proliferation assay kit was

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purchased from Solarbio. Reagents such as antibodies were purchased from Abcam, Santa Cruz Biotechnology and NOVUS: p-SRC (ab40660, Abcam), SRC (ab109381, Abcam), p-Vav2 (ab86695, Abcam), Vav2 (ab52640, Abcam), p-p66SHC (ab68166, Abcam), p66SHC (ab33770, Abcam), GAPDH (ab8245, Abcam), Prohibitin (sc-377037, Santa Cruz Biotechnology), NOX4 (NB110-58849, NOVUS), GFAP (ab7260, Abcam). RAC1 activity assay kit was purchased from Cell Biolabs. NOX activity assay kit, MDA (malondialdehyde) assay kit and SOD (super oxide dismutase) activity assay kit were purchased from Solarbio and Abcam. Other chemicals and reagents used in this study were obtained from Beyotime and Sangon.

2.2 Cell culture and treatment

Enteric glial cell line CRL-2690 (ATCC, VA, U.S.A.) was cultured in Dulbecco minimum essential medium (DMEM) with 10% fetal bovine serum (FBS) and 5.6 mM D-glucose. Hyperglycemic stress was achieved by adding D-glucose to the concentration of 200 mM. For the deactivation of redoxosomes, cells were pre-treated with RAC1 inhibitor (NSC23766, 80 μM, 6 h) or NOX inhibitor (VAS2870, 10 μM, 6 h) for 12 h, and then treated with high glucose (200 mM) for another 24 h. For the knockdown of p66SHC, cells were transfected with p66SHC siRNA (100 nM, GenePharma) using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocols for 48 h, and then treated with high glucose (200 mM) for another 24 h.

2.3 STZ-induced diabetic mice

Wild-type C57BL/6J mice ($n = 6$ per group) from Changzhou Cavens Experimental Animal Center (Changzhou, China) were induced by intraperitoneal injection of STZ (50 mg/kg in 10 mM citrate buffer, pH 4.5) injection for 5 consecutive days. Control mice ($n = 6$ per group) were administered with sodium citrate buffer. The blood from tail vein was collected for the measurement of glucose level. The mice with a blood glucose level of over 300 mg/dL were considered as DM. After collection of blood samples, these animals were kept in individual metabolic cages for 24 h and food consumption and drinking water (distilled water) per 24 h were measured.

2.4 MTT assay

Cell viability was determined using the MTT assay [18,19]. After the indicated treatment of high glucose (200 mM) for 24 h, cells were incubated with 0.5 mg/mL MTT solution (100 μL) for 3 h at 37°C. The cultured medium was then replaced with DMSO (dimethyl sulfoxide) solution (150 μL) and the absorbance was recorded at a wavelength of 490 nm (DTX800; Beckman Coulter, Inc.) according to the manufacturer's protocols.

2.5 TUNEL assay

Cell death was determined using the TUNEL (Terminal Deoxynucleotidyl Transferase Mediated dUTP Nick End Labeling) assay [20]. It is a laboratory technique used to detect DNA fragmentation, based on the ability of the terminal deoxynucleotidyl transferase (TdT) enzyme to add deoxynucleotides to the 3′ ends of DNA strands that have been nicked. For cells, 100 μL/well of the permeabilization reagent (0.2% Triton X-100 in PBS/phosphate buffer saline) was added after the fixation for 10 min at room temperature. For tissues, sections were deparaffinized, digested with Proteinase K (Beyotime). Cell or tissue samples were processed using the TUNEL kit (Beyotime) and visualized with a fluorescent microscope (Olympus DP73, Olympus).

2.6 Oxidative marker analysis

Intracellular ROS generation was assessed using a DCFH-DA-based ROS assay kit (Solarbio) [21]. Briefly, cells were incubated with DCFH-DA (2′,7′-Dichlorodihydrofluorescein diacetate, 10 μM) for 15 min at 37°C, and ROS concentration (normalized intensity) was analyzed by measuring fluorescence using a fluorescence microplate reader (SpectraMax M5, Molecular Devices). For MDA measurement, the samples from cell lysates or tissues were processed using the TBA (thiobarbituric acid) method (Abcam). For SOD activity measurement, the samples from cell lysates or tissues were processed using NBT (nitro blue tetrazolium) reaction methods (Abcam).

2.7 NOX activity analysis

NOX activity was determined by luminescence assay using lucigenin as the electron acceptor generated by the NADPH (nicotinamide adenine dinucleotide phosphate) oxidase complex [22]. Cells were sonicated and the lysates (250 μg/ml of protein) were incubated with 20 μM lucigenin and 100 μM NADPH. The emitted luminescence was detected by the luminometer (FluoStar Optima, BMG Labtech).

2.8 Western blot analysis

Cells were treated with RIPA (radio immune-precipitation assay) lysis buffer and protein concentrations were determined by BCA (bicinchoninic acid) assay [23]. Samples (20 μg) were added to 10% of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, and proteins were electrophoresed. Following electrophoresis, total proteins were transferred to polyvinylidene fluoride (PVDF) membrane and membranes were then proceeded with primary antibody and secondary antibody. The protein bands were visualized using the ECL (electrochemiluminescence) assay kit.

2.9 Immunofluorescence analysis

Cells or tissue sections after treatment were incubated with primary antibody overnight at 4°C, and then were washed and incubated with fluorescence-conjugated secondary antibody for 1 h. After a washing step with PBS, samples were incubated with ProLong gold Antifade reagent containing DAPI (4,6-Diamidino-2-phenylindole) for visualization of nuclei. Immunofluorescence images were captured by microscopy (Olympus DP73, Olympus).

2.10 Intestinal intramuscular injection

Mice were anesthetized using isoflurane (2–4%) and kept at a constant body temperature. For the experiments, the

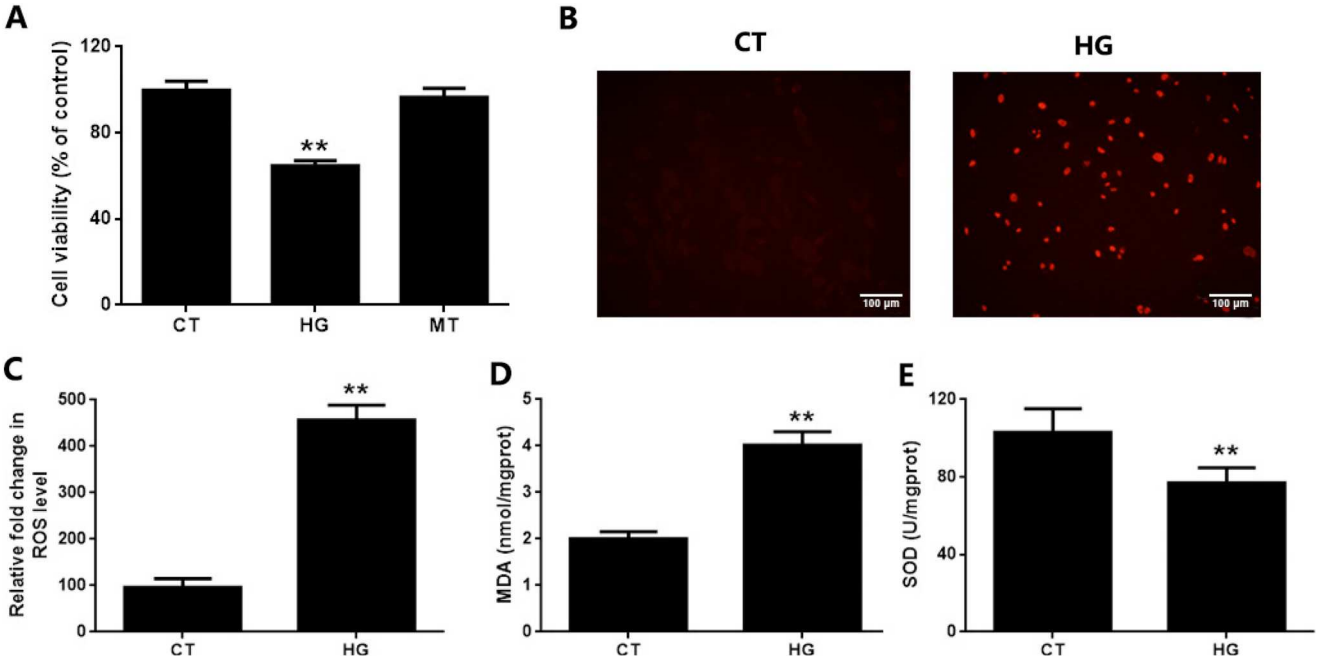

Figure 1. Hyperglycemic stress induces oxidative damage of EGCs *in vitro*. CRL-2690 cells were treated with high glucose (200 mM) or mannitol (200 mM) for 24 h. **B:** Cell viability was assessed using MTT assay. **C:** Cell apoptosis was assessed using TUNEL assay. **D:** ROS generation was evaluated using DCFH-DA staining. **E:** MDA level of cell lysates was measured using TBA assay. **F:** SOD activity of cell lysates was investigated using NBT assay. ** $p < 0.01$ *vs.* CT. CT: control (5.6 mM), HG: high glucose (200 mM), MT: mannitol (200 mM).

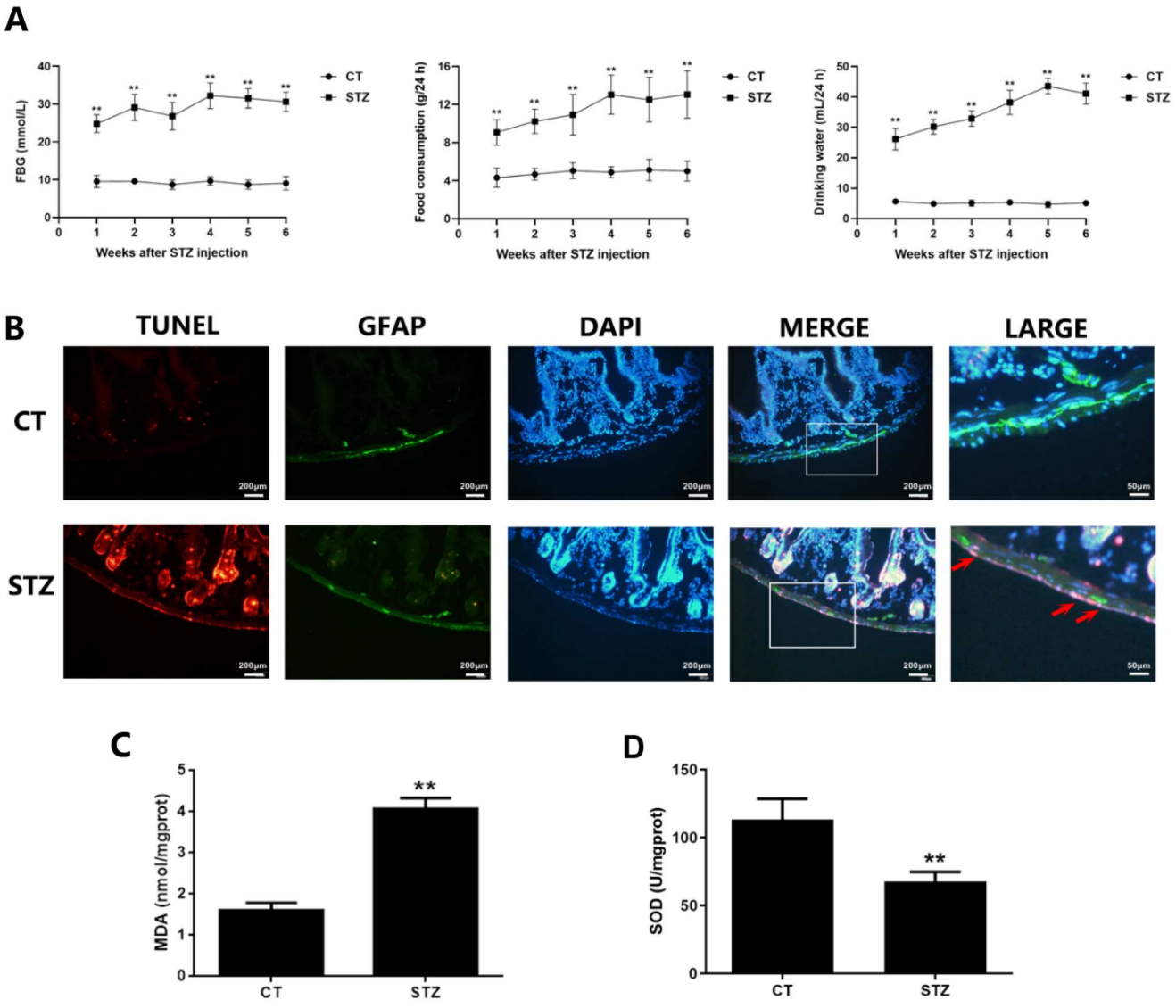

Figure 2. Hyperglycemic stress induces oxidative damage in EGCs *in vivo*. A: Fast blood glucose (FBG), food consumption and drinking water (distilled water) per 24 h were measured in CT and DM mice. **B:** Cell apoptosis of EGCs in the duodenum of CT and DM mice was analyzed using immunofluorescence analysis (TUNEL: red fluorescence, GFAP: green fluorescence, DAPI: blue fluorescence). **C&D:** The samples of duodenum tissues were collected from CT and DM mice. MDA level of tissues was assessed using TBA assay and SOD activity of tissues was studied using NBT assay. ** $p < 0.01$ vs. CT. CT: control (<100 mg/dL); STZ: DM (>300 mg/dL) (6 weeks post STZ-injection).

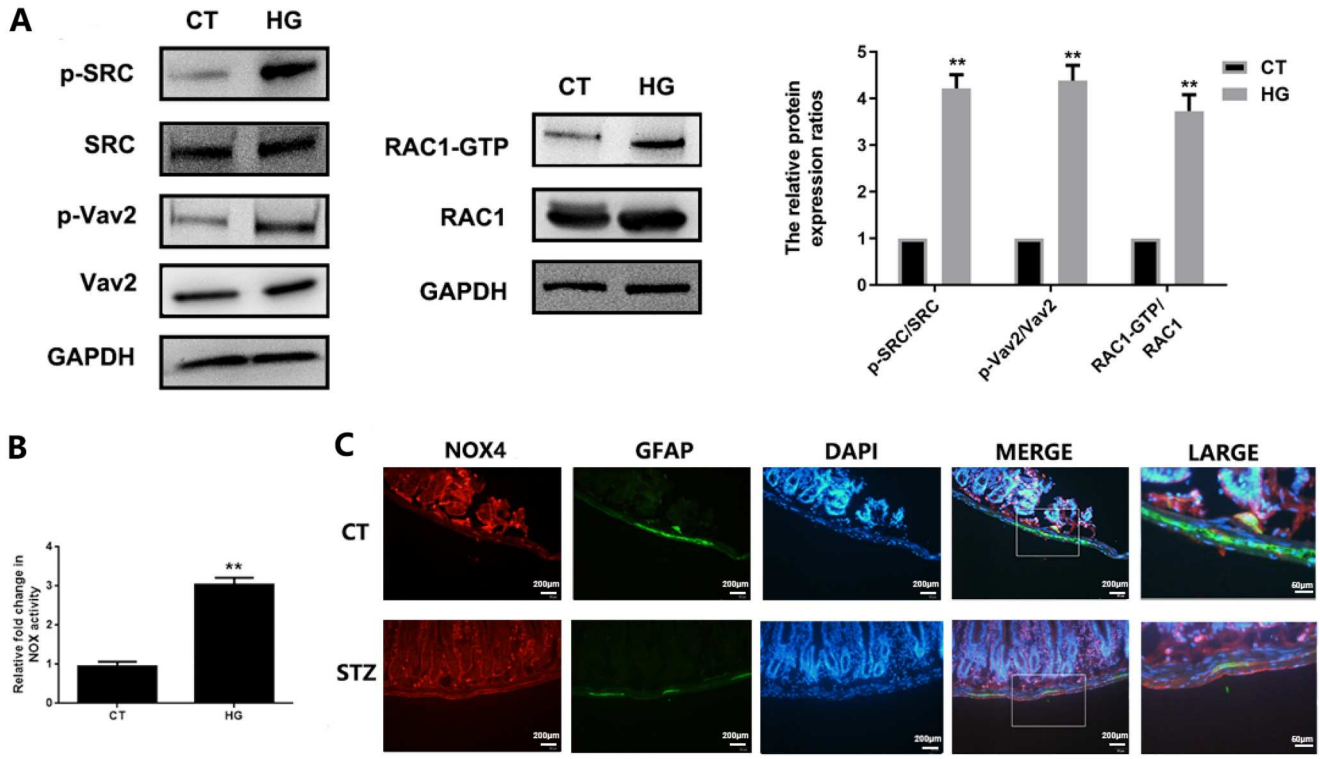

Figure 3. Hyperglycemic stress induces redoxosomes activation in EGCs *in vitro* and *in vivo*. A&B: CRL-2690 cells were treated with high glucose (200 mM) for 6 h or 12 h. The expressions of redoxosomes-related proteins were assessed using western blot analysis. NOX activity was evaluated using DCPIP assay. CT: control (5.6 mM), HG: high glucose (200 mM). **C:** The samples of duodenum tissues were collected from CT and DM mice. The expressions of NOX4 in EGCs were assessed using immunofluorescence analysis (NOX4: red fluorescence, GFAP: green fluorescence, DAPI: blue fluorescence). ** $p < 0.01$ vs. CT. CT: control (<100 mg/dL); STZ: DM (>300 mg/dL) (6 weeks post STZ-injection).

injection of compounds or the AAV (adeno-associated virus) packaging system was conducted using a 10 μL Hamilton syringe into the wall of the duodenum at 2 sites, near the myenteric plexus, as previously reported [9,24,25].

2.11 Statistical analysis

SPSS19.0 software was used to analyze data. The data were expressed as means ± SD. Statistical comparisons were made by Student's *t*-test between two groups and one-way

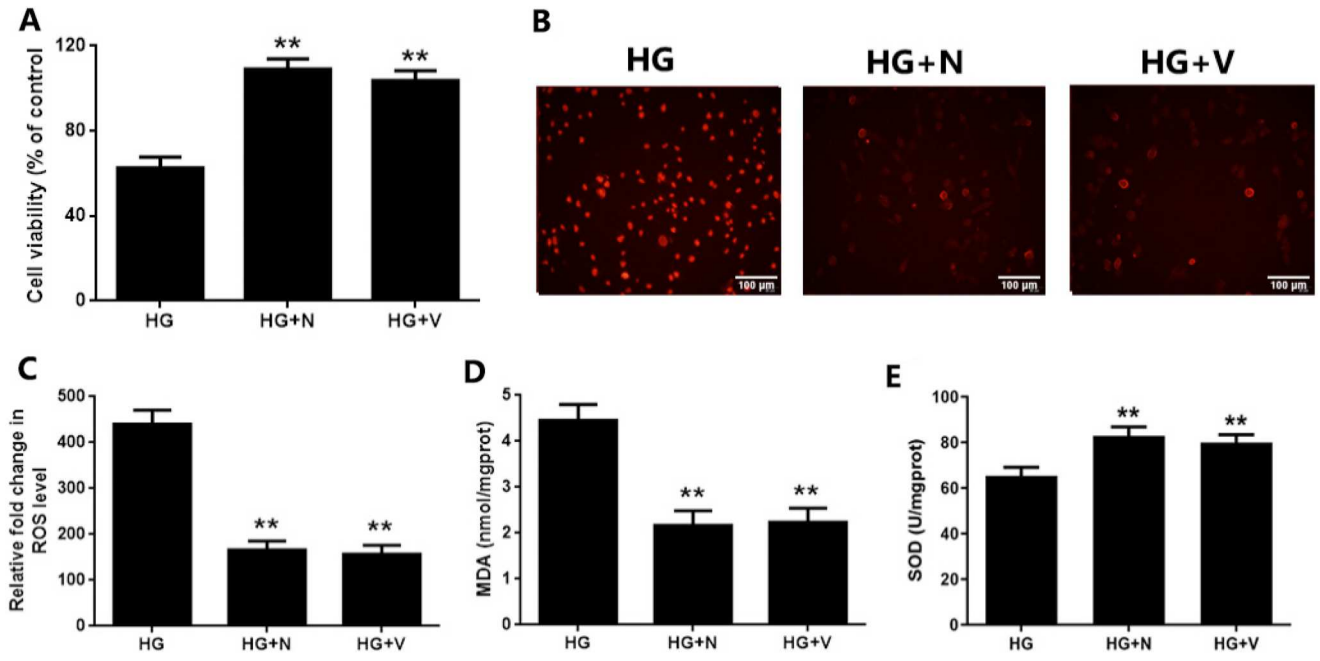

Figure 4. The *in vitro* redoxosomes deactivation attenuates EGCs oxidative damage under hyperglycemic stress. CRL-2690 cells were pre-treated with RAC1 inhibitor (NSC23766, 80 μM, 6 h) or NOX inhibitor (VAS2870, 10 μM, 6 h) for 12 h, and then treated with high glucose (200 mM) for another 24 h. **A:** Cell viability was assessed using MTT assay. **B:** Cell apoptosis was evaluated using TUNEL assay. **C:** ROS generation was investigated using DCFH-DA staining. **D:** MDA level of cell lysates was assessed using TBA assay. **E:** SOD activity of cell lysates was measured using NBT assay. ** $p < 0.01$ vs. HG. CT: control (5.6 mM), HG: high glucose (200 mM).

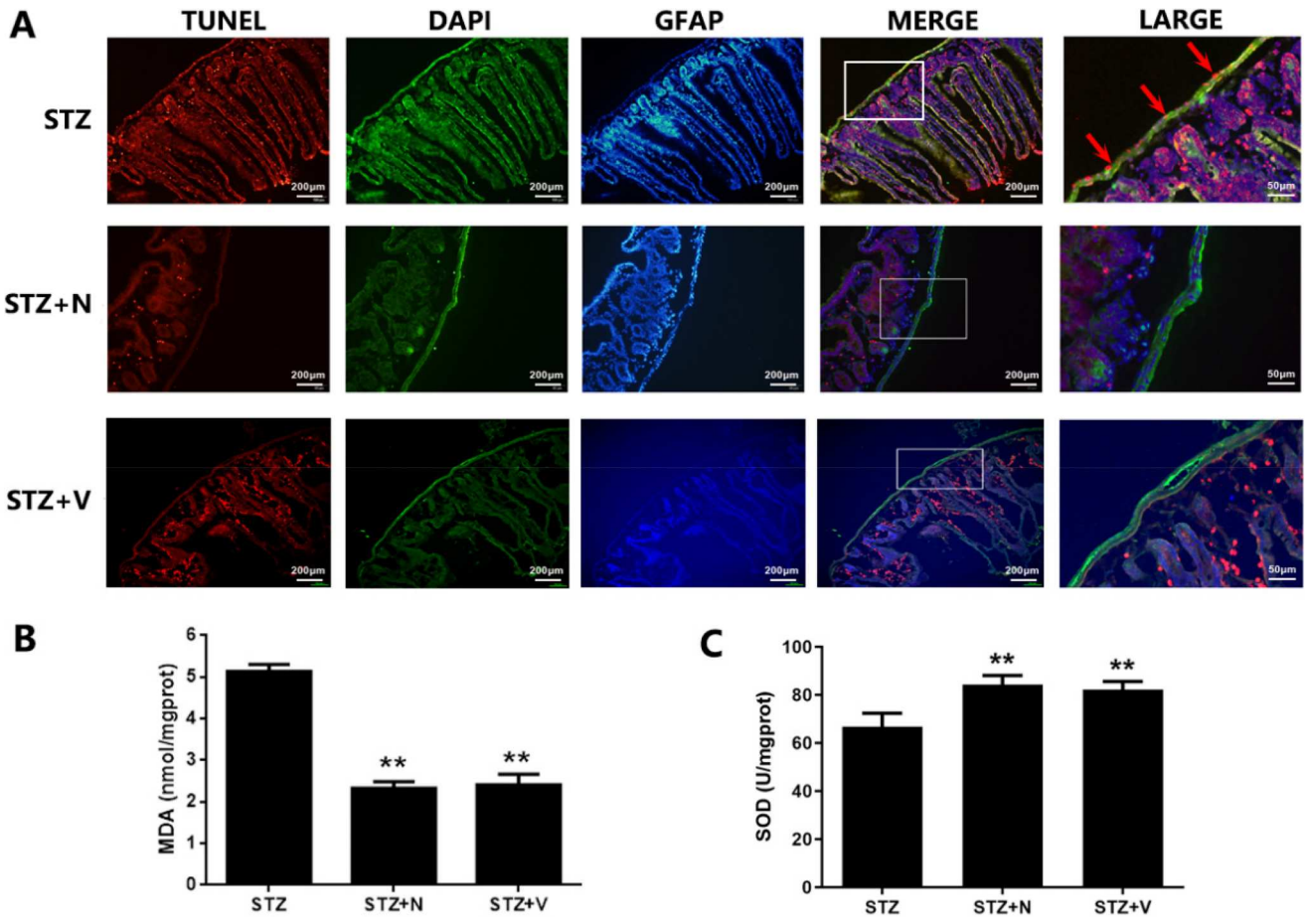

Figure 5. The *in vivo* redoxosomes deactivation ameliorates EGCs oxidative damage in hyperglycemic stress. Diabetic mice (2 weeks post STZ-injection) were intestinal intramuscular injected with NSC23766 (150 μM/every 3 d/4 weeks) or VAS2870 (50 μM/every 3 d/4 weeks). The apoptosis of EGCs in duodenum of DM mice was analyzed using immunofluorescence analysis (TUNEL: red fluorescence, GFAP: green fluorescence, DAPI: blue fluorescence). **B&C:** The samples of duodenum tissues were collected from CT and DM mice. MDA level of tissues was assessed using TBA assay and SOD activity of tissues was evaluated using NBT assay. ** $p < 0.01$ vs. CT. CT: control (<100 mg/dL); STZ: DM (>300 mg/dL) (6 weeks post STZ-injection).

ANOVA followed by Tukey's post hoc test among multiple groups. $P < 0.05$ indicated statistically significant differences.

3. Results

3.1 Hyperglycemic stress induces oxidative damage in *EGCs* in vitro *and* in vivo

The impact of hyperglycemic stress on cell viability of EGCs was evaluated using *in vitro* (CRL-2690 cells) and *in vivo* (C57BL/6J mice) models. Hyperglycemia significantly reduced cell viability and induced cell apoptosis by increasing ROS generation and MDA levels and reducing SOD activities, in contrast to normal glucose *in vitro* (Figure 1). In addition, mannitol treatment (200 mM) did not result in a significant alteration of cell viability, indicating the observed cell apoptosis was caused by high glucose instead of high permeability. Diabetic mice with hyperglycemia at 6 weeks post STZ injection showed the increased cell apoptosis of EGCs in the duodenum, with the increased MDA levels and the decreased SOD activities, in comparison with normal mice *in vivo* (Figure 2).

3.2 Hyperglycemic stress induces redoxosomes activation in *EGCs* in vitro *and* in vivo

The regulatory mechanism of hyperglycemic stress on EGCs was further evaluated in relation to oxidative stress-related signaling. Hyperglycemic stress significantly induced redoxosomes activation by triggering the phosphorylation of SRC tyrosine and Vav2 tyrosine and increasing active RAC1-GTP expression and NOX activity (Figure 3A&B). Diabetic mice with hyperglycemia at 6 weeks post STZ injection showed the increased NOX4 expression (red fluorescence) in EGCs of the duodenum (Figure 3C).

3.3 Redoxosomes inactivation attenuates hyperglycemic stress-induced EGCs oxidative damage

To explore the role of redoxosomes in hyperglycemic stress-induced cytotoxicity in EGCs, RAC1 inhibitor (NSC23766) or NOX inhibitor (VAS2870) was applied to deactivate redoxosomes under hyperglycemic stress. NSC23766 or VAS2870 pre-treatment attenuated hyperglycemic stress-induced cytotoxic effect in EGCs, via decreasing ROS generation and MDA level and increasing SOD activity (Figure 4). Diabetic mice receiving intestinal intramuscular injection of NSC23766 or VAS2870 showed the decreased cell apoptosis in EGCs of the duodenum (Figure 5). Such effects were observed with the decreased MDA level and the increased SOD activity in duodenum tissues. In addition, treatment of NSC23766 or VAS2870 did not affect FBG, feed and water consumption of diabetic mice (data not shown).

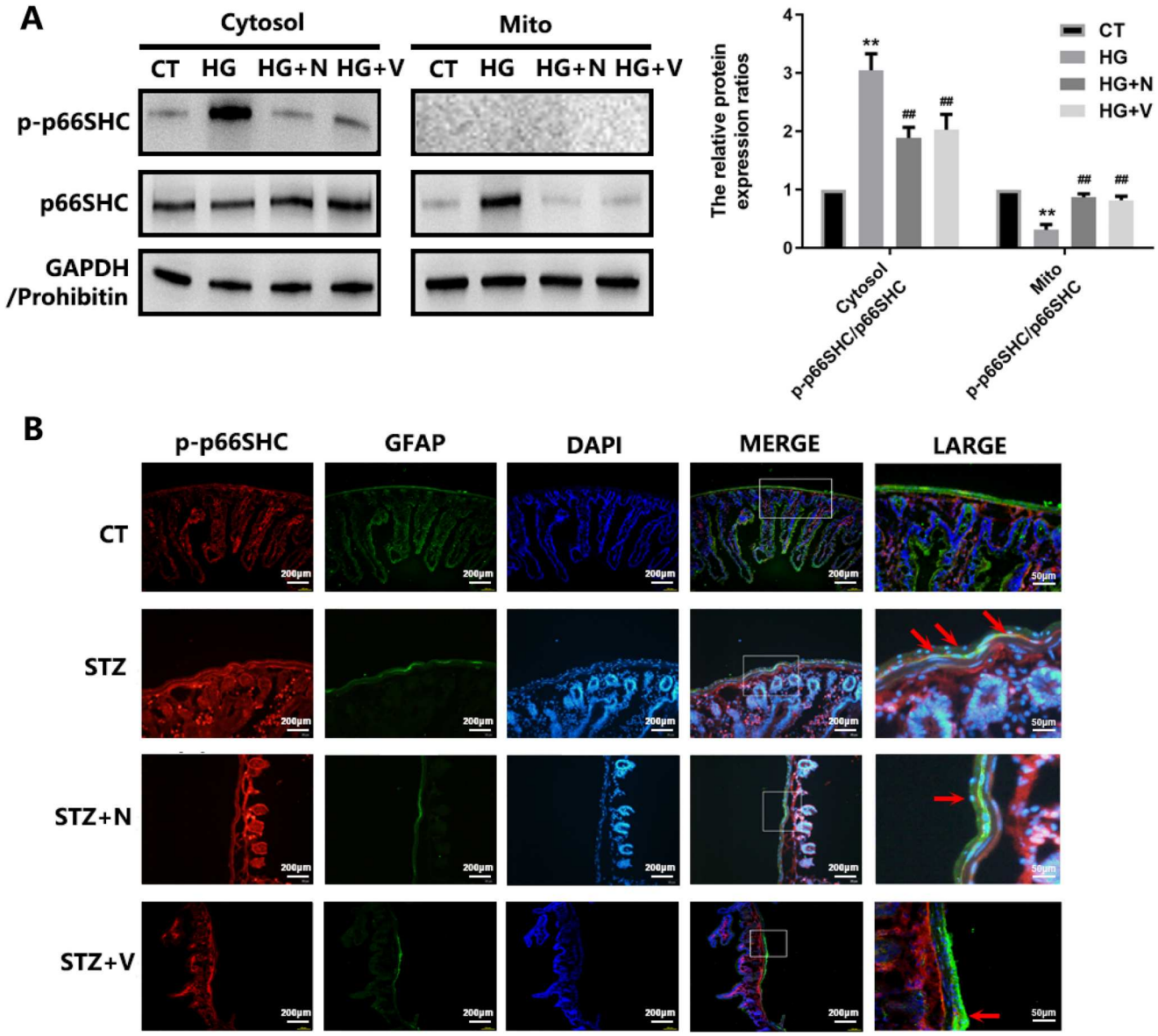

Figure 6. Hyperglycemic stress induces p66SHC activation in EGCs *in vitro* and *in vivo*. A: CRL-2690 cells were pre-treated with RAC1 inhibitor (NSC23766, 80 μM, 6 h) or NOX inhibitor (VAS2870, 10 μM, 6 h) for 12 h, and then treated with high glucose (200 mM) for another 12 h. The protein expression and distribution of p-p66SHC and p66SHC were evaluated using western blot analysis. CT: control (5.6 mM), HG: high glucose (200 mM). **B:** Diabetic mice (2 weeks post STZ-injection) were intramuscularly injected with NSC23766 (150 μM/every 3 d/4 weeks) or VAS2870 (50 μM/every 3 d/4 weeks). The expression of p-p66SHC in EGCs were assessed using immunofluorescence analysis (p-p66SHC: red fluorescence, GFAP: green fluorescence, DAPI: blue fluorescence). ** $p < 0.01$ vs. CT. ## $p < 0.01$ *vs.* HG. CT: control (<100 mg/dL); STZ: DM (>300 mg/dL) (6 weeks post STZ-injection).

3.4 Hyperglycemic stress induces redoxosomes activation in EGCs by activating p66SHC

As reported in the previous study, p66SHC is one of the downstream targets of redoxosomes under hyperglycemic stress. The expression and distribution of p-p66SHC were assessed in this present study., Hyperglycemic stress induced serine36 phosphorylation and mitochondrial translocation of p66SHC (Figure 6A&B). However, RAC1 inhibitor (NSC23766, 80 μM, 6 h) or NOX inhibitor (VAS2870, 10 μM, 6 h) pre-treatment blocked such effects. Diabetic mice at 6 weeks post STZ injection showed the increased p-p66SHC (phosphorylated-p66SHC) in EGCs at duodenum, which was attenuated by intestinal intramuscular injection of NSC23766 or VAS2870 (Figure 6C).

3.5 p66SHC deactivation attenuates hyperglycemic stress-induced oxidative damage in EGCs

Knockdown of p66SHC attenuated hyperglycemic stress-induced cytotoxic effect in EGCs, via decreasing ROS generation and MDA level as well as increasing SOD activity (Figure 7). Diabetic mice with p66SHC knockdown showed the reduced cell apoptosis of EGCs in the duodenum, which was accompanied by a decreased MDA level and increased SOD activity in duodenum tissues (Figure 8).

4. Discussion

Until now, studies of DGD are still lacking. Enteric glial cells (EGCs) found in the gastrointestinal tract are confirmed to be involved in the process of gastrointestinal dysfunction under hyperglycemic stress. Li *et al* have reported that T2DM mice show the induced colonic inflammation, changes in the gut microbiota and dysfunction of EGCs [26]. Our previous study has revealed that hyperglycemic stress induced EGCs injury in both *in vitro* and *in vivo* DM models, *via* modulating BTK/NF-κB/IL-1α/IL-10 signaling [27]. In this study, we found that hyperglycemia significantly reduced cell viability and induced cell apoptosis in EGCs by

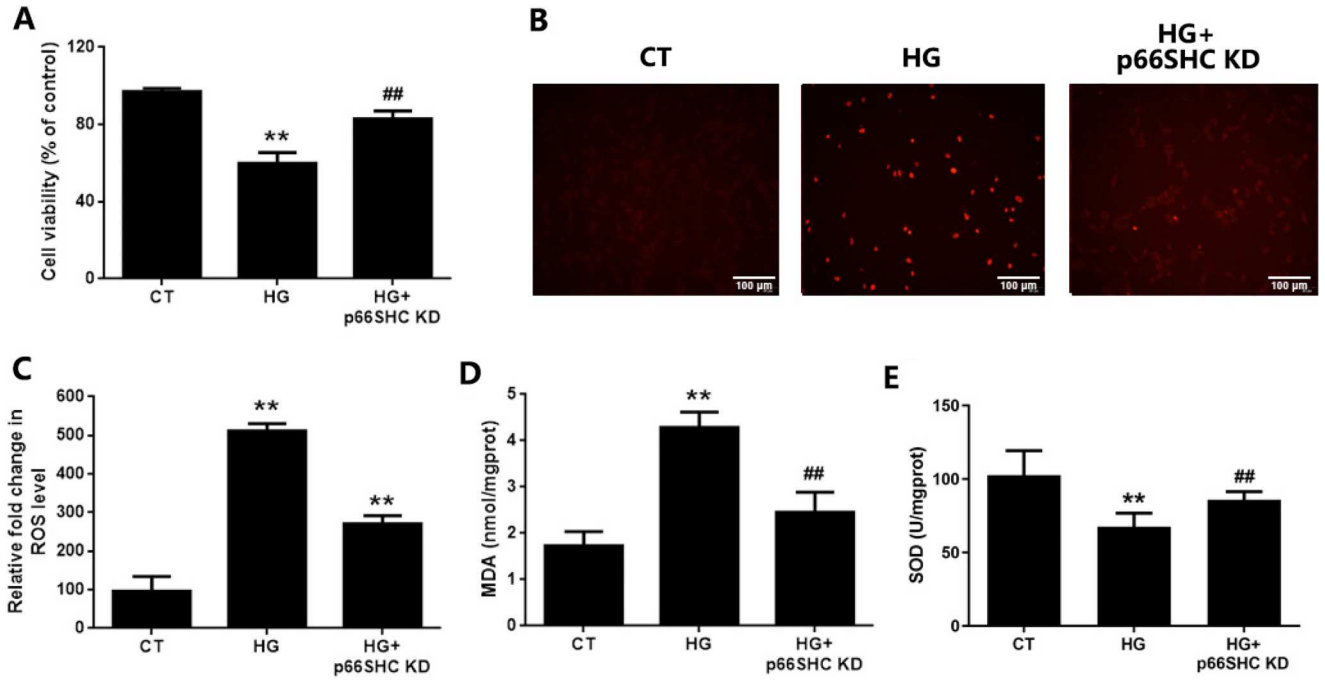

Figure 7. The *in vitro* p66SHC knockdown ameliorates oxidative damage of EGCs under hyperglycemic stress. CRL-2690 cells with p66SHC knockdown were treated with high glucose (200 mM) for 24 h. **A:** Cell viability was assessed using MTT assay. **B:** Cell apoptosis was evaluated using TUNEL assay. **C:** ROS generation was investigated using DCFH-DA staining. **D:** MDA level of cell lysates was assessed using TBA assay. **E:** SOD activity of cell lysates was studied using NBT assay. ** $p < 0.01$ vs. CT, ## $p < 0.01$ vs. HG. CT: control; HG: high glucose; KD: knockdown.

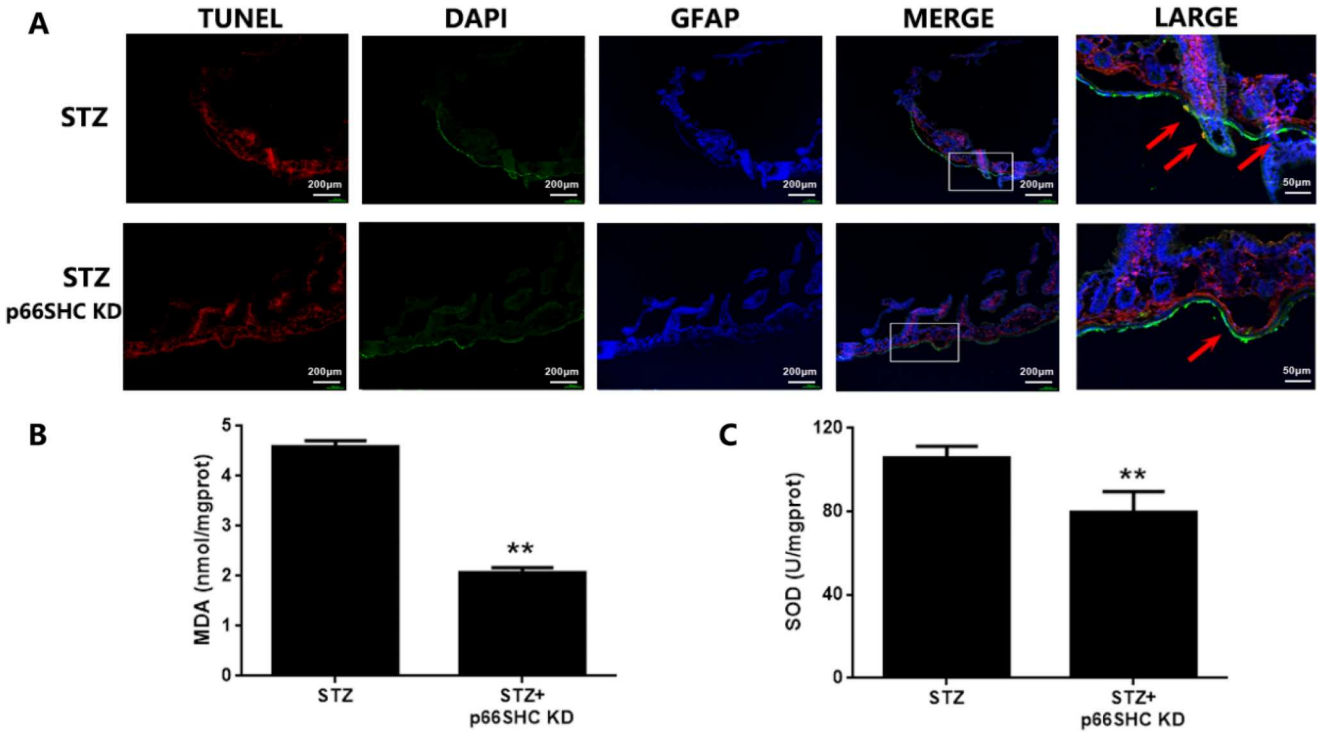

Figure 8. The *in vivo* p66SHC knockdown ameliorates oxidative damage in EGCs under hyperglycemic stress. Mice were intestinal-intramuscular injected with AAV-9-p66SHC shRNA for 7 days and then injected with STZ for 5 days. **A:** Cell apoptosis of EGCs in the duodenum of DM mice was analyzed using immunofluorescence analysis (TUNEL: red fluorescence, GFAP: green fluorescence, DAPI: blue fluorescence). **B&C:** The samples of duodenum tissues were collected from CT and DM mice. MDA level of tissues was assessed using TBA assay and SOD activity of tissues was assessed using NBT assay. ** $p < 0.01$ vs. DM. CT: control; STZ: DM (6 weeks post STZ-injection).

increasing oxidative stress, *in vitro* and *in vivo*. And, the detailed mechanism responsible for this effect was further investigated.

Redoxosomes mediate the transmission of ROS signals from the endosome interior to redox-sensitive effectors on the endosomal surface in response to environmental stimuli [28]. The previous studies indicate that hyperglycemic stress is a critical stimulator of redoxosomes activation in rat retinal capillary endothelial cells (TR-iBRB2) and renal mesangial cells, leading to diabetes-related complications [15,16].

Till now, the role of redoxosomes in hyperglycemic stress-induced oxidative damage in EGCs has not been elucidated. In the present study, we found that hyperglycemic stress-induced oxidative damage in EGCs was dependent on redoxosomes activation, *via* inducing phosphorylation of SRC and Vav2, up-regulation of active RAC1-GTP expression and NOX activity. Furthermore, redoxosomes inhibitors (RAC1 inhibitor: NSC23766, NOX inhibitor: VAS2870) potently attenuated EGCs dysfunction under hyperglycemic stress. In addition, treatment of NSC23766 or VAS2870 did not affect FBG, feed and water consumption of diabetic mice.

p66SHC, a member of SHC family proteins, is an oxidative stress sensor and regulator in cells. p66SHC is capable of initiating MAP-kinase signaling and activating the c-fos promoter, distinct from p52Shc and p46Shc [29]. Accumulating evidence indicated that p66SHC acts as a novel biomarker in determining development and progression of chronic age-related diseases, neoplasms, and metabolic diseases such as diabetes [30]. Yohei *et al* have reported that p66SHC-mediated inflammatory cascade leads to oxidative stress and is a causative pathogenic mechanism in diabetes-associated cognitive impairment [31]. Mattia *et al* have shown that p66SHC activation is also associated with diabetes-associated myelopoiesis. Thus, targeting p66SHC may be a new therapeutic strategy in these diseases [32]. Oxidative injury in EGCs contributes to the pathological process of DGD. In the present study, we are the first to report that p66SHC is an effector of redoxosomes and redoxosomes-dependent p66SHC activation leads to excessive ROS generation and EGCs oxidative stress in *in vitro* and *in vivo* DM models. However, there are still some limitations in this study, and future research is warranted to investigate the function of EGCs in the other sites of gastrointestinal tract in DM models.

5. Conclusion

In conclusion, our data indicates the important role of redoxosomes-p66SHC signaling in the oxidative damage of EGCs during the pathological process of DGD. Therefore, such signaling cascade may be a potential therapeutic target in the treatment of DGD.

Acknowledgements

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The authors confirm that the datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethical approval

All protocols used for animal manipulation were approved by the Institutional Animal Care Committee.

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