

The role of p66Shc in high glucose concentration-induced oxidative damage in R28 cells

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Abstract

Purpose: To investigate the effect of high glucose concentrations on the expression of p66Shc and phosphorylation of p66Shc Ser36 in R28 cells, and to elucidate the role of p66Shc in high glucose-induced oxidative damage and apoptosis of R28 cells. **Methods:** R28 cells were cultured with various concentrations of D-glucose, or with 30 mM D-glucose or 30 mM mannitol only for the indicated timepoints. The expression of p66Shc and phosphorylation of p66Shc Ser36 were subsequently detected. Additionally, R28 cells were divided into five groups: i. control (CTL) group; ii. high glucose (HG) group; iii. high glucose p66Shc Wt (HG p66Shc Wt) group; iv. high glucose p66Shc siRNA (HG p66Shc siRNA) group; and v. high glucose p66Shc S36A (HG p66Shc S36A) group. P66Shc, cytochrome C, and reactive oxygen species (ROS) content in mitochondria, mitochondrial membrane potential levels, mitochondrial DNA damage, and apoptosis were also evaluated. **Results:** With the increase of glucose concentration, the levels of p66Shc mRNA and expression of p66Shc protein gradually increased. P66Shc mRNA and protein levels, and phosphorylation of p66Shc Ser36 increased with the prolongation of exposure to high concentrations of glucose. Notably, after stimulation with 30 mM mannitol, the levels of p66Shc mRNA, p66Shc protein, and p66Shc S36 phosphorylation did not change. With the increase of p66Shc level in the mitochondria of R28 cells, ROS content in mitochondria increased, mitochondrial membrane potential decreases, mitochondrial DNA damage was exacerbated, cytochrome C content in mitochondria decreased, and apoptosis increased. **Conclusion:** High glucose concentrations increased the expression of p66Shc in R28 cells in a time- and concentration-dependent manner. P66Shc is an essential regulator of mitochondrial ROS, and play an important role in mitochondrial apoptosis.

Background

In mammals, there are three Shc genes, called Shc A, Shc B, and Shc C, respectively. The Shc A gene can produce three proteins, namely p46Shc, p52Shc and p66Shc. Compared with the other two proteins, p66Shc has an additional proline-rich structure (CH2) at the N-terminal, in which amino acid 36 contains a phosphorylatable serine residue (S36) and a cytochrome C domain, both of which are necessary for p66Shc protein phosphorylation and its mediation of reactive oxygen species (ROS) production and mitochondrial-induced apoptosis (1).

P66Shc is an important adaptor protein. It regulates the life cycle of mammalian cells and oxidative stress. It is also associated with oxidative damage in cells. P66Shc can induce an increase in ROS content of cells, thus affecting the rate of oxidative damage, promoting apoptosis and regulating cell viability. Previous studies have shown that deletion of the p66Shc gene alleviates endothelial dysfunction and oxidative damage which are induced by hyperglycemia (2, 3). In the kidney tissue of SD rats and db/db mice (a type 2 diabetes mellitus mouse model), the expression of p66Shc was increased significantly (4). In contrast, in Akita mice with p66Shc gene deletion, the oxidative stress response was significantly reduced, glomerular and renal tubule damage was alleviated, and urinary albumin was decreased (4). P66Shc gene deletion can also attenuate tissue damage (5), vascular cell apoptosis (6),

and ROS-mediated age-dependent endothelial dysfunction (7). Wu et al. (8) found that inhibiting the expression of p66Shc in retinal pigment epithelial cells can reduce oxidative damage and apoptosis.

Our previous study found that with the development of diabetes mellitus, both the p66Shc mRNA and p66Shc protein increased gradually (9). TUNEL results showed that correlating with the expression of p66Shc, the apoptotic cells' number increased gradually (9). These results suggest that p66Shc may cause the occurrence and development of diabetic retinopathy. Understanding the role and mechanism of p66Shc in the progression of diabetic retinopathy may be helpful for the early treatment of diabetic retinopathy.

R28 retinal precursor cell line originated from rat retina culture on the 6th day after birth. With adenovirus 12S E1A (NP-040507) gene in replication deficiency virus vector, the retina was immortalized. Various retinal cell behavioral studies, such as neuroprotection, cytotoxicity, differentiation and photostimulation, as well as retinal neurological function and gene expression, can use R28 cells, both *in vitro* and *in vivo* (10). Although cell culture is not completely equivalent to complete eyes, many studies of retinal processes still used R28 cells as an important experimental system (10).

The correct characterization of cell lines is extremely important (12). In the past, we thought that the RGC-5 cell line was a rat retinal ganglion cell line. But now we find that it is actually an unrelated SV40 transformed mouse photoreceptor cell line 661W (11). In order to avoid the same situation, strict cell culture schemes must be followed, and published experiments on the identity of R28 cells must be validated. The origin and identity of R28 cells were supported by several published studies (13, 14). The function of R28 cells was studied. The results showed that R28 cells had retinal neurotransmitter receptors and could respond to the stimulation of neurotransmitters (15, 16). Through *in vitro* studies of R28 cells, many important findings which were related to retinal function and specific disease status have been found (17, 18).

The aim of this study was to investigate the expression of p66Shc and phosphorylated p66Shc S36 in R28 cells under high glucose condition, and to clarify the role of p66Shc in oxidative damage and apoptosis of R28 cells under high glucose condition.

Materials And Methods

R28 cell culture and treatment

R28 cells were purchased from Cell Resources Center, Shanghai Academy of Life Sciences. They were cultured in glucose-free DMEM at 37°C in a 5% CO₂ environment, supplemented with 10% fetal bovine serum (FBS), 1×10⁵ U/L penicillin, and 100 mg/L streptomycin until 80% of the cells fused. Cells were then incubated in DMEM medium without FBS for 24 hours. Subsequently, R28 cells were cultured with various concentrations of D-glucose (5.5, 15, 30, and 45 mM). After 12 hours of glucose stimulation, the levels of p66Shc mRNA in R28 cells were detected by RT-PCR. Western blot was then used to detect the expression of p66Shc protein in R28 cells after 24 hours of glucose stimulation. Meanwhile, R28 cells

were cultured with 30 mM D-glucose or 30 mM D-mannitol for the indicated time. The levels of p66Shc (after 0, 6, 12, 24 hours of intervention) and phosphorylation of p66Shc S36 (after 0, 15, 30, 60, 90, 120, and 180 min of intervention) were detected by Western blotting, respectively.

Concurrently, R28 cells were divided into five groups: i. control (CTL) group (untransfected R28 cells + 5.5 mM glucose); ii. high glucose group (HG) (untransfected R28 cells + 30mM glucose); iii. high glucose p66Shc wild type (HG p66Shc Wt) group (R28 cells transfected with p66Shc wild type + 30mM glucose); iv. high glucose p66Shc siRNA (HG p66Shc siRNA) group (R28 cells transfected with small interfering RNA for p66Shc + 30mM glucose); v. high glucose p66Shc S36A (HG p66Shc S36A) group (R28 cell transfected with p66Shc S36A mutant + 30mM glucose).

The production of ROS by R28 cells was evaluated by labeling cells with MitoSox followed by detection by laser confocal microscopy at 24 hours after glucose stimulation. The mitochondrial membrane potential levels were measured using a laser confocal microscope at 24 hours of group intervention. Flow cytometry was used to detect apoptosis at 48 hours after glucose stimulation. Western blot was used to detect the expression of p66Shc and cytochrome C in mitochondria after 48 hours of high glucose intervention. PCR was used to detect the mitochondrial DNA damage after 48 hours of high glucose intervention.

Transfection

Cells were transfected with p66Shc wild type (WT), the p66Shc S36A mutant, or the small interfering RNA for p66Shc (all obtained from Biofort Biotechnology Co., LTD, Shanghai, China) using LIPOFECTAMINE 2000 reagent (Invitrogen, Carlsbad, CA). After 6 hours of transfection, the medium was changed and replaced with a plasmid/lipo-free mixed solution to obtain stable expression cells, and continued to expand the culture. The efficiency of overexpression and inhibition was confirmed by Western blot.

Real time PCR

We used The Trizol method to extract RNA. P66Shc primer was designed by Primer Premier 5.0 software and synthesized by Invitrogen Biotechnology Co., Ltd. (Shanghai, China). The primer sets are listed in Table 1. We mixed the sample with 1 ml Trizol (Invitrogen Life Technologies, Shanghai, China) and shake it well, then put it on ice for about 5 minutes to denaturate it completely. After that, we mixed the mixture with 0.25 ml chloroform, shake for 15 seconds, and stand for 3 minutes at 4 C, centrifuged the mixture at $12,000 \times g$ for 10 min, and collected the supernatant. Then, we added 0.5 ml isopropanol, mixed, and let the mixture rest for 10 minutes at room temperature, followed by centrifuged the mixture for 10 minutes at $12,000 \times g$, and discarded the supernatant. After cleaning the precipitated particles, we added 1.5 ml 75% ethanol, centrifuged the mixture for 8 minutes at $12,000 \times g$. We removed the supernatant, and air-dried the precipitated RNA for 5-10 minutes. The Revert Aid First Strand cDNA Synthesis Kit (Thermo, Shanghai, China) was used to reverse transcribe total RNA (2 μ g), according to the manufacturer's protocols. GAPDH was used as the internal reference gene. Amplification of 20 μ l reaction mixture was

carried out with the following PCR program: 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 60 s. We used $2^{-\Delta\Delta Ct}$ method to analyze the data.

Western blot

RIPA lysis buffer containing protease inhibitors was used to extract protein. We mixed the protein sample with the sample buffer, heated it in 100°C water for 10 minutes, and then cooled the mixture in ice water. Then we loaded the proteins (10~20 g) onto a sodium dodecyl sulphate (SDS) 12% polyacrylamide gel. After electrophoresis, the protein was transferred onto polyvinylidene fluoride membranes. 5% skimmed milk in 0.1% Tween/Tris-buffered saline (TBST) was used to block the nonspecific binding. Membranes were incubated with primary antibodies against p66Shc (1:1000; Abcam, Shanghai, China), serine 36 phosphorylated p66Shc (1:500; Abcam, Shanghai, China), and GAPDH (1:5000; Boster, Shanghai, China) overnight at 4°C. Then we used phosphate buffered saline containing Tween (PBST) to wash the membrane three times in a shaker. After that, we immersed the membrane in the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. We washed the membrane three times again in PBST, then visualized the protein band with enhanced chemiluminescence reaction kit (Goodbio Biological Technology Co., LTD, Wuhan, China), and captured the protein band with Tanon 5500 imager (Tanon, Shanghai, China). We analyzed the band with AlphaEaseFC (Alpha Innotech, San Leandro, CA, USA). Each band is normalized according to the corresponding GAPDH band.

MitoSOX Red

The production of ROS by R28 cells was evaluated by labeling cells with MitoSox (Invitrogen) according to the manufacturer instructions. MitoSOX Red is a kind of reactive cell permeable dye. It can target mitochondria rapidly and selectively, and is mainly used to observe mitochondrial O₂ levels. MitoSOX red fluorescence (excitation at 510 nm and emission at 585 nm) was detected by laser scanning confocal microscopy equipped with a bandpass filter (Zeiss LSM 510; Carl Zeiss, Thornwood, NY, USA).

Mitochondrial membrane potential levels

Mitochondrial membrane potential can reflect cells damage sensitively. Under normal conditions, the mitochondrial membrane potential is about 1.0-2.0 mV, and the intramembrane potential is lower than that in the cytoplasm. When the mitochondrial membrane is damaged, the membrane potential decreases. In this study, the mitochondrial membrane potential levels were measured using a laser confocal microscopy equipped with a bandpass filter (Zeiss LSM 510; Carl Zeiss, Thornwood, NY, USA) after 24 hours of group intervention. Briefly, cells were incubated with TMRE (membrane potential) at 37 °C for 30 min. DAPI stained with nucleus and washed three times with PBS for 5 min/time.

Flow cytometry

We used an annexin V-FITC (fluorescein isothiocyanate) Kit (Dojindo, Shanghai, China) to quantify the percentage of undergoing apoptotic cells. Briefly, R28 cells were divided into the groups as above-mentioned. Then the cells were inoculated into 6-well plates. After 48 hours of treatment, the cells were

digested by trypsin, then centrifuged at 1000 rpm at 4 °C for 5 minutes, washed by PBS, and incubated with a mixture of 5 µl annexin V-FITC conjugate and 5 µl propidium iodide (PI) at room temperature in darkness for 15 minutes. A flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) was used to detect apoptosis.

Isolation of mitochondria

Mitochondria were prepared by using Thermo Fisher mitochondria isolation Kit (Pierce, Rockford, IL, USA) (19). Briefly, after digesting the homogenized samples with the kit reagents, the samples were centrifuged for 10 minutes at $700 \times g$, then centrifugation for 15 minutes at $3000 \times g$. The pellet thus obtained was washed, suspended in PBS, and used for the quantification of p66Shc and cytochrome C in mitochondria.

Mitochondrial DNA damage

Mitochondrial DNA is sensitive to oxidative damage as it lacks repair mechanisms. When the aggregation and replication of mitochondrial DNA are inhibited, the number of long copies of mitochondrial DNA decreases, suggesting mitochondrial DNA damage. Detecting the ratio of long fragments to short fragments of mitochondrial DNA can thus reflect the integrity of mitochondrial DNA (20).

Primer sequences for mitochondrial DNA damage was designed by Primer Premier 5.0 software and synthesized by Invitrogen Biotechnology Co., Ltd. (Shanghai, China). The primer sets are listed in Table 2. Briefly, long (8.8 kb) and short (223 bp) mtDNA regions were amplified using PCR, and the amplified products were resolved on an agarose gel. Relative amplification was quantified by normalizing the intensity of the long product to the short product (8.8 kb/223 bp).

Statistical analysis

We used the Statistical Package for Social Sciences (version 11.0, SPSS Inc., Chicago, IL, USA) to perform statistical analysis. The mean \pm standard deviation (SD) expressed data. The difference between two groups used the Student's t-test to analyze. The differences among groups used One-way ANOVA to analyze. $P < 0.05$ was considered a significant difference.

Results

Effect of transfection

Western blot was used to detect the expression of p66Shc protein in R28 cells after 48 hours of transfection. The results showed that the expression of p66Shc in the p66Shc Wt group and p66Shc S36A group was significantly higher than that of the control group ($P < 0.05$, $P < 0.05$). However, compared with the control group, the expression of p66Shc in p66Shc siRNA group was significantly decreased ($P < 0.05$) (Figure 1).

Effect of different concentrations of glucose on the expression of p66Shc in R28 cells

R28 cells were cultured with various concentrations of D-glucose (5.5, 15, 30, and 45 mM). After 12 hours of glucose stimulation, the levels of p66Shc mRNA in R28 cells were detected by RT-PCR. The results showed that with the increase in glucose concentration, the level of p66Shc mRNA increased gradually, and was the greatest in the 45 mM glucose treatment group. Notably, there was a significant difference among the groups (ANOVA, $P < 0.05$). Western blot was then used to detect the expression of p66Shc protein in R28 cells after 24 hours of glucose stimulation. Interestingly, normal R28 cells expressed a baseline level of p66Shc. With the increase of glucose concentration, its expression gradually increased and was also the greatest in the 45 mM glucose treatment group. Again, there was a significant difference among the groups (ANOVA, $P < 0.05$) (Figure 2a, 2b).

Changes in p66Shc expression over time after glucose stimulation

R28 cells were stimulated by 30mM glucose or 30 mM mannitol. The level of p66Shc mRNA was detected by PCR, and the expression of p66Shc protein was detected by Western blot, although at different time points (0, 6, 12, 24 hours). Importantly, the intracellular level of p66Shc mRNA and protein both increased gradually over time in high glucose group (ANOVA, $P < 0.05$, and $P < 0.05$ respectively) (Figure 3a, 3b). In contrast, in the isotonic control group (30 mM mannitol), the expression of p66Shc mRNA and protein did not change significantly with prolonged mannitol exposure time, and there was no significant difference among the groups (ANOVA, $P > 0.05$) (Figure 3c, 3d).

Changes in p66Shc Ser36 phosphorylation over time after glucose stimulation

R28 cells were stimulated by 30mM glucose or 30 mM mannitol. The phosphorylation of p66Shc Ser36 in R28 cells at different time points ((0, 15, 30, 60, 90, 120, and 180 min)) was detected by Western blot. The results showed that the phosphorylation of p66Shc Ser36 was significantly increased after 15 minutes of glucose treatment compared with the control group (0 h group) ($P < 0.05$). With the prolongation of glucose exposure time, the protein level increased gradually, and there was a significant difference among the groups (ANOVA, $P < 0.05$). In contrast, the level of phosphorylated Ser36 p66Shc protein in R28 cells stimulated with isotonic mannitol (30 mM) did not change significantly with the prolongation of treatment time (ANOVA, $P > 0.05$) (Figure 4a, 4b).

Detection of mitochondrial ROS content by laser scanning confocal microscopy

We used MitoSox Red to detect the fluorescence generated by mitochondrial ROS. R28 cells were incubated with 5 μ m MitoSox Red and 2 μ g/ml Hoechst for 10 minutes at 37°C. MitoSox Red can penetrate the living cell membrane freely and enter the cell. It acts selectively on the mitochondria and is oxidized by the ROS in the mitochondria. It shows red fluorescence under confocal laser microscopy. Nucleus shows blue fluorescence. After glucose stimulation for 24 hours, the level of red fluorescence was weak in the normal control group, while it was significantly increased in the HG group ($P < 0.05$), and was further increased in the HG p66Shc Wt group (compared with the HG group, $P < 0.05$). In contrast, compared with the HG group, the red fluorescence (ROS content) of HG p66Shc siRNA group and HG p66Shc S36A group decreased significantly ($P < 0.05$, $P < 0.05$) (Figure 5a, 5b).

Detection of mitochondrial membrane potential levels by laser confocal microscopy

Using a laser confocal microscopy, the mitochondrial membrane potential was red fluorescent and the nucleus was blue fluorescent. After glucose stimulation for 24 hours, compared with the normal control group, the red fluorescence expression was significantly decreased in the HG group ($P < 0.05$), and was further decreased in the HG p66Shc Wt group (compared with the HG group, $P < 0.05$). The red fluorescence expression of the HG p66Shc siRNA group and the HG p66Shc S36A group recovered partially, compared with the HG group, the difference was significant ($P < 0.05$, $P < 0.05$). (Figure 5a, 5c)

R28 cells apoptosis

After 48 hours of glucose intervention, a flow cytometry was used to detect apoptosis. Compared with the normal control group, the apoptosis rate was significantly higher in the HG group ($P < 0.05$). Among all the groups, the rate of apoptosis was the highest in the HG p66Shc Wt group, and there was a significant difference between it and the HG group ($P < 0.05$). However, compared with the HG group, the apoptosis rate in the HG p66Shc siRNA group and the HG p66Shc S36A group were significantly decreased ($P < 0.05$ and $P < 0.05$, respectively) (Figure 6). These results suggest that the high glucose induced R28 cells apoptosis was regulated by p66Shc.

Expression of p66Shc and cytochrome C in mitochondria

After 48 hours of high glucose intervention, mitochondrial proteins were extracted from each group, and the expression of p66Shc and cytochrome C in mitochondria detected by Western blot. The results showed that the expression of p66Shc in the mitochondria of the HG group was significantly higher than that of the normal group ($P < 0.05$). Among all the groups, the expression of p66Shc was the highest in the HG p66Shc Wt group, and there was a significant difference between it and the HG group ($P < 0.05$). However, compared with the HG group, the expression of p66Shc in the HG p66Shc siRNA group was significantly decreased ($P < 0.05$). There was no significant difference in the expression of p66Shc in mitochondria between the HG p66Shc S36A group and the HG group ($P > 0.05$) (Figure 7a).

In contrast, the expression of cytochrome C in mitochondria in the HG group was significantly lower than that in normal group ($P < 0.05$). Among all the groups, the expression of cytochrome C in mitochondria was the lowest in the HG p66Shc Wt group, and there was a significant difference between it and the HG group ($P < 0.05$). However, compared with the HG group, the expression of cytochrome C in mitochondria in the HG p66Shc siRNA group and the HG p66Shc S36A group were both significantly increased ($P < 0.05$) (Figure 7b).

Detection of mitochondrial DNA damage by PCR

The integrity of mitochondrial DNA (mtDNA) reflects the overall damage to mtDNA. After 48 hours of glucose intervention, mtDNA was extracted from cells of each group, and the integrity of mtDNA was detected by PCR. Compared with the normal group, the proportion of long fragments of mitochondrial DNA in the HG group was decreased significantly ($P < 0.05$). The proportion of long fragments of

mitochondrial DNA decreased further in the HG p66Shc Wt group ($P < 0.05$). However, this was attenuated in the HG p66Shc siRNA group and the HG p66Shc S36A group ($P < 0.05$, $P < 0.05$ respectively) (Figure 8).

Discussion

Diabetic retinopathy is one of the serious microvascular complications in type 1 and type 2 diabetes mellitus. Many risk factors are associated with the occurrence and development of diabetic retinopathy, such as hypertension, proteinuria, and hyperlipidemia (21). Previous studies have shown that under high glucose conditions, the production of ROS increases, which in turn results in the accumulation of extracellular matrix and apoptosis (22). P66Shc has an oxidoreductase activity, and it can directly stimulate mitochondrial ROS generation (23, 24). In Cai et al.'s study (24), they cultured human embryonic kidney cells under high glucose conditions and found that the expression of p66Shc increased with glucose concentration, which suggested that p66Shc may be involved in the pathogenesis of diabetic nephropathy. Inactivation of p66Shc would increase the resistance to oxidative stress and protect mice from diabetic or age-related vascular endothelial dysfunction (2, 7).

In this study, we found that the levels of p66Shc mRNA and protein both increased with increasing glucose concentration. Furthermore, at the same concentration of glucose, their levels increased gradually with prolonged incubation time. To exclude the effect of osmotic pressure on the expression of p66Shc in R28 cells, we used 30 mM mannitol as a control group. The results showed that with prolonged incubation time, the expression of p66Shc mRNA and protein did not change significantly in the mannitol group, in agreement with the results of Sun et al. (25). This indicated that high glucose concentration did not regulate the expression of p66Shc through the osmotic pathway. However, the precise mechanism of how high glucose concentration regulates p66Shc expression remains unclear. Previous studies have shown that diabetes increases presynaptic signaling (26) through a variety of mechanisms, including hyperglycemia, aging, oxidative stress, and the NF- κ B response. Presynaptic signals induce the increase of cytosolic and mitochondrial p66Shc pools, serine phosphorylation, mitochondrial pool release from a high-molecular weight complex, cytochrome C oxidation, and proapoptotic H₂O₂ production. In addition, with the increase of electron flow from complex III, cytochrome C decreases excessively. It is speculated that if cytochrome C is excessively reduced, the reaction with cytochrome C may occur. Hypoxia or nitric oxide inhibition (up-regulation in diabetic state) results in a decrease in cytochrome C oxidase activity, followed by an increase in electron flow from complex III. Diabetes also increases the electron flow of complex III. P66Shc mediates oxidant-induced apoptosis and regulates the levels of steady-state ROS, which therefore would reflect the moderate activation of p66Shc through chronic stress. Moreover, in diabetes mellitus, this effect of p66Shc could be enhanced by inducing persistent excess of ROS in mitochondria or activation of cytosolic NAD(P)H oxidase (27-29).

In this study, we found that with the increase of p66Shc level in mitochondria of R28 cells, ROS content in mitochondria increased, mitochondrial membrane potential decreases, mitochondrial DNA damage was exacerbated, cytochrome C content in mitochondria decreased, and apoptosis increased. These results

suggested that p66Shc plays an important role in mitochondrial apoptosis. Under high glucose conditions, p66Shc produced a marked amount of ROS in mitochondria, increased the permeability of the mitochondrial membrane, released cytochrome C, and activated caspase-9 in the cytoplasm, inducing apoptosis (30, 31).

The main site of ROS production in cells is the mitochondria. Mitochondrial DNA is a ring-shaped bare structure without self-repair mechanisms, thus it is very susceptible to oxidative damage (32). Brownlee et al. (33) suggested that the increase of ROS production in mitochondria is a common mechanism of diabetic complications. Increased ROS will activate vascular injury pathways (polyol pathway activation, advanced glycation end products, protein kinase C (PKC) activation, and aminohexose pathway), stimulate oxidative stress responses in tissues, and cause mitochondrial DNA damage and dysfunction of electron transfer chain complexes, which in turn leads to excessive ROS production.

We treated R28 cells with high concentrations of glucose. After 15 minutes, the phosphorylation of p66Shc Ser36 was significantly increased. With the prolongation of treatment time, the phosphorylation also increased gradually. However, there was no significant change in p66Shc Ser36 phosphorylation in mannitol treated R28 cells. In the high glucose p66Shc S36A group, the ROS content and the apoptosis rate were both attenuated. These results suggested that the phosphorylation of p66Shc Ser36 in R28 cells may play an important role in the oxidative damage induced by high glucose. The key to p66Shc mediated oxidative stress injury is Ser36 phosphorylation (34-37). High glucose will stimulate the phosphorylation of p66Shc Ser36 in the cytoplasm. The phosphorylated p66Shc is recognized and isomerized by prolyl isomerase pin1, and then dephosphorylated by protein phosphatase 2A, enters the mitochondria, oxidizes cytochrome C in the mitochondria, and eventually produces ROS, which will cause extracellular matrix accumulation and apoptosis (38). Vono et al. (39) found that PKC- β promoted the phosphorylation of p66Shc Ser36 in skeletal muscle of patients with diabetes, and inhibiting PKC- β decreased the phosphorylation of p66Shc Ser36 and promoted the recovery of skeletal muscle in patients with diabetes. Song et al. (40) also found that the PKC- δ pathway regulated the activation and transport of p66Shc in renal tubular cells in diabetic nephropathy. PKC siRNA blocks high glucose concentration-induced p66Shc phosphorylation and mitochondrial translocation, which reduces the production of ROS in renal tubular cells. A PKC- β inhibitor (LY333531) reduced the occurrence and development of diabetic retinopathy (19, 41, 42). However, in diabetic retinopathy, whether p66Shc Ser36 phosphorylation is also through the PKC- β pathway, or alternative pathways, remains to be elucidated.

Conclusions

High glucose concentrations increased the expression of p66Shc in R28 cells in a time and concentration dependent manner. Under high glucose conditions, p66Shc causes oxidative damage and apoptosis in R28 cells, which is increased by overexpression of p66Shc. In contrast, p66Shc siRNA or p66Shc S36A mutation decreases oxidative damage and apoptosis in R28 cells induced by high glucose concentrations. In summary, present study suggests that p66Shc is an essential regulator of mitochondrial ROS, and play an important role in mitochondrial apoptosis. The study will provide a novel

insight into the mechanism associated with increased mitochondrial dysfunction in the development of diabetic retinopathy.

Abbreviations

FBS: fetal bovine serum

SDS: sodium dodecyl sulphate

FITC: fluorescein isothiocyanate

PI: propidium iodide

SD: standard deviation

mtDNA: mitochondrial DNA

PKC: protein kinase C

CTL: control

KO: knockout

RT-PCR: Reverse transcription-polymerase chain reaction

DM: diabetes mellitus

DR: Diabetic retinopathy

ROS: reactive oxygen species

STZ: streptozotocin

RIPA: radio-immunoprecipitation assay

TBST: Tween/Tris-buffered saline

PBST: phosphate buffer solution Tween

PBS: phosphate buffer saline

Declarations

Ethics approval and consent to participate

The study was approved by the Shanghai Jiaotong University affiliated No.6 hospital.

Availability of data and materials

The datasets used and analysed during the current study available from the corresponding author on reasonable request.

Consent for publication

Not Applicable.

Competing interests

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Authors' contributions

PR Lu conceived of the study, and participated in its design. MH Zhao participated in the design of the study, and mitochondrial DNA damage, and apoptosis evaluation. JY Hu participated in immunoassay and performed the statistical analysis. Q Wu participated in immunoassay. All authors read and approved the final manuscript.

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Tables

Table 1. The PCR primers

Primer	Sequence (5'-3')
P66Shc-F	GAAGAGCCACCTGACCATC
P66Shc-R	AGGCACCCGAAGAGCATC
GAPDH-F	ACAAC TTTGGTATCGTGGAAGG
GAPDH-R	GCCATCACGCCACAGTTTC

Table 2. The PCR primers

Primer	Sequence (5'-3')
short fragments of mitochondrial DNA-F	CGACAGCTAAGACCCAAACTGGG
short fragments of mitochondrial DNA-R	CCCATTTCTTCCCATTTCATTGGC
long fragments of mitochondrial DNA-F	TACTAGTCCGCGAGCCTTCAAAGC
long fragments of mitochondrial DNA-R	GGGTGATCTTTGTTTGCGGGT

Figures

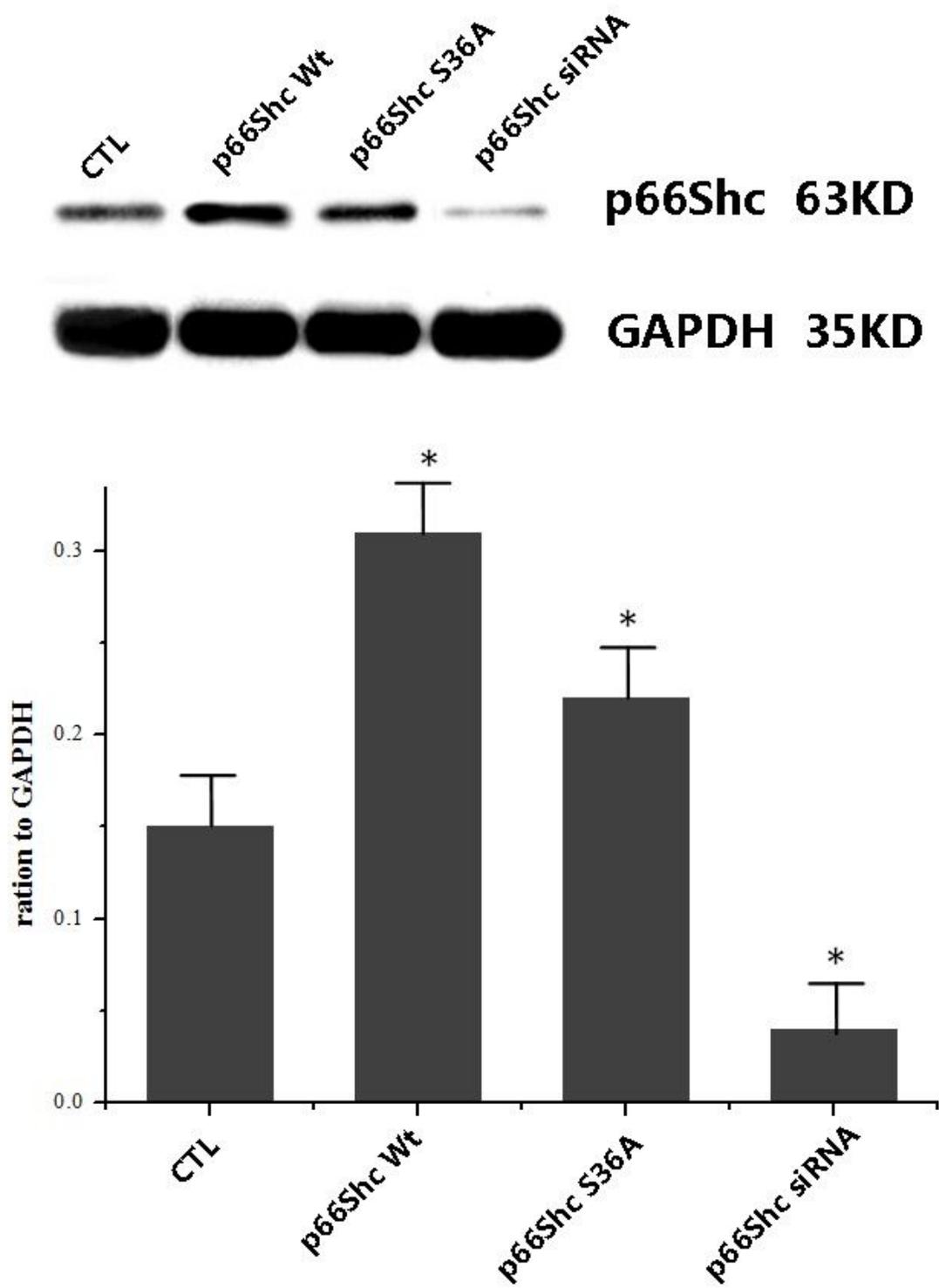


Figure 1

Western blot was used to detect the expression of p66Shc protein in R28 cells after 48 hours of transfection. Compared with the control group, the expression of p66Shc in the p66Shc Wt group and p66Shc S36A group was significantly higher; however, the expression of p66Shc in p66Shc siRNA group was significantly decreased. (*: compared with the control group, $P < 0.05$).

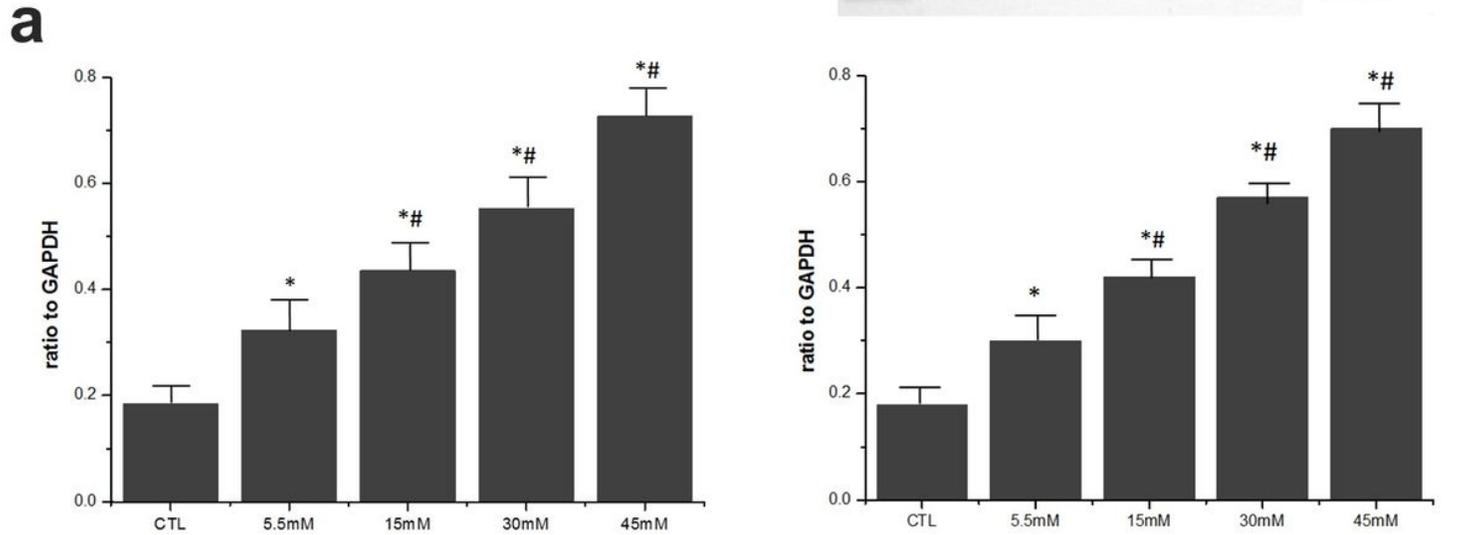


Figure 2

(a) RT-PCR was used to detect the expression of p66Shc in R28 cells after 12 hours of glucose stimulation. With the increase in glucose concentration, the level of p66Shc mRNA increased gradually. There was a significant difference among the groups. (b) Western blot was used to detect the expression of p66Shc protein in R28 cells after 24 hours of glucose stimulation. With the increase of glucose concentration, its expression gradually increased. There was a significant difference among the groups. (*: compared with the normal control group, $P < 0.05$; #: compared with the 5.5mM group, $P < 0.05$).

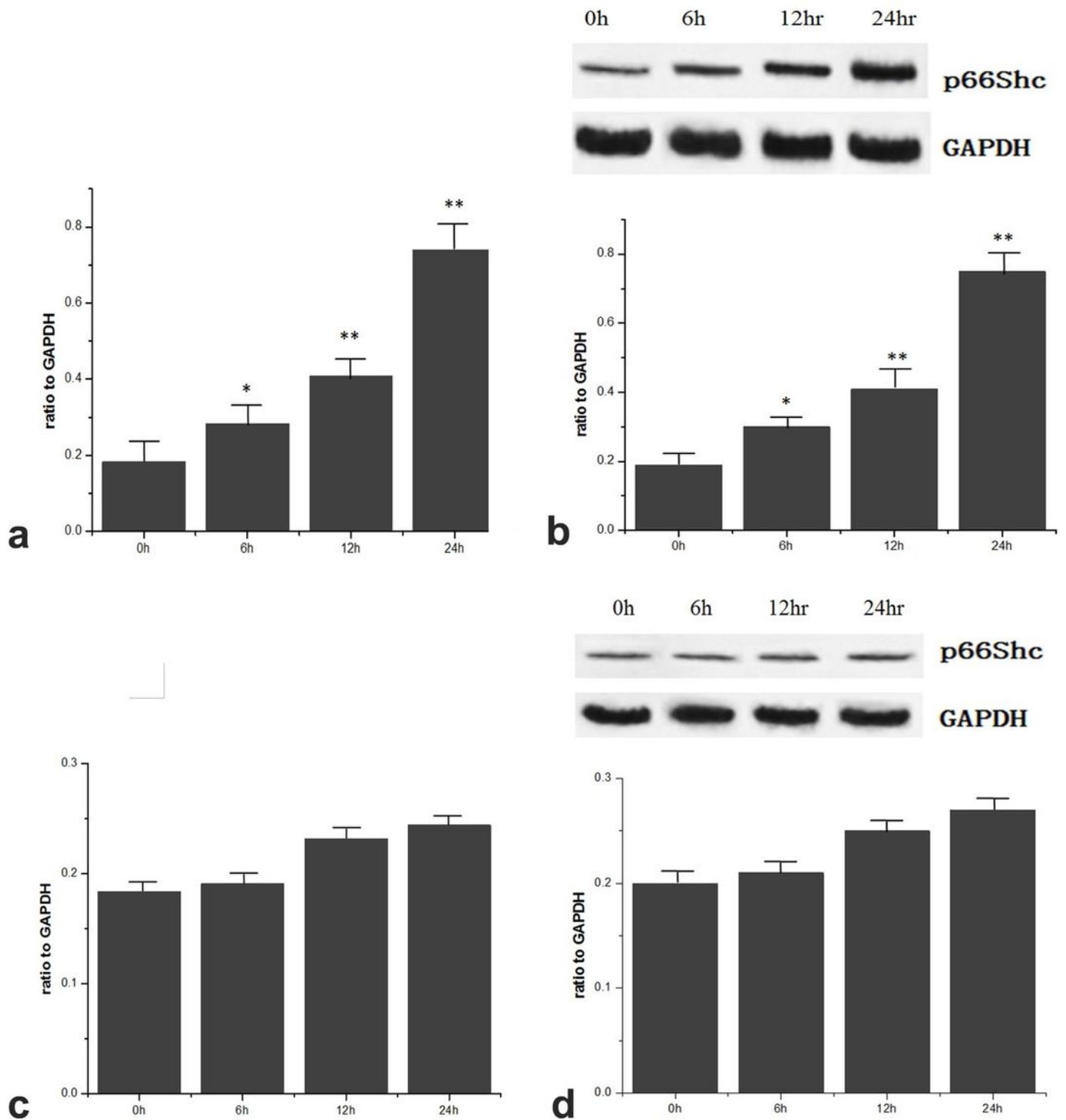


Figure 3

(a) R28 cells were stimulated by 30mM glucose. The level of p66Shc mRNA was detected by PCR at different time points. The intracellular level of p66Shc mRNA increased gradually over time. (b) R28 cells were stimulated by 30mM glucose. The expression of p66Shc protein was detected by western blot at different time points. The intracellular level of p66Shc protein increased gradually over time. (c) 30mM mannitol stimulated R28 cells. The expression of p66Shc mRNA did not change significantly with

prolonged mannitol exposure time, and there was no significant difference among the groups. (d) 30mM mannitol stimulated R28 cells. Protein did not change significantly with prolonged mannitol exposure time, and there was no significant difference among the groups. (*: compared with the group 0h, $P < 0.05$; **: compared with the group 0h, $P < 0.01$).

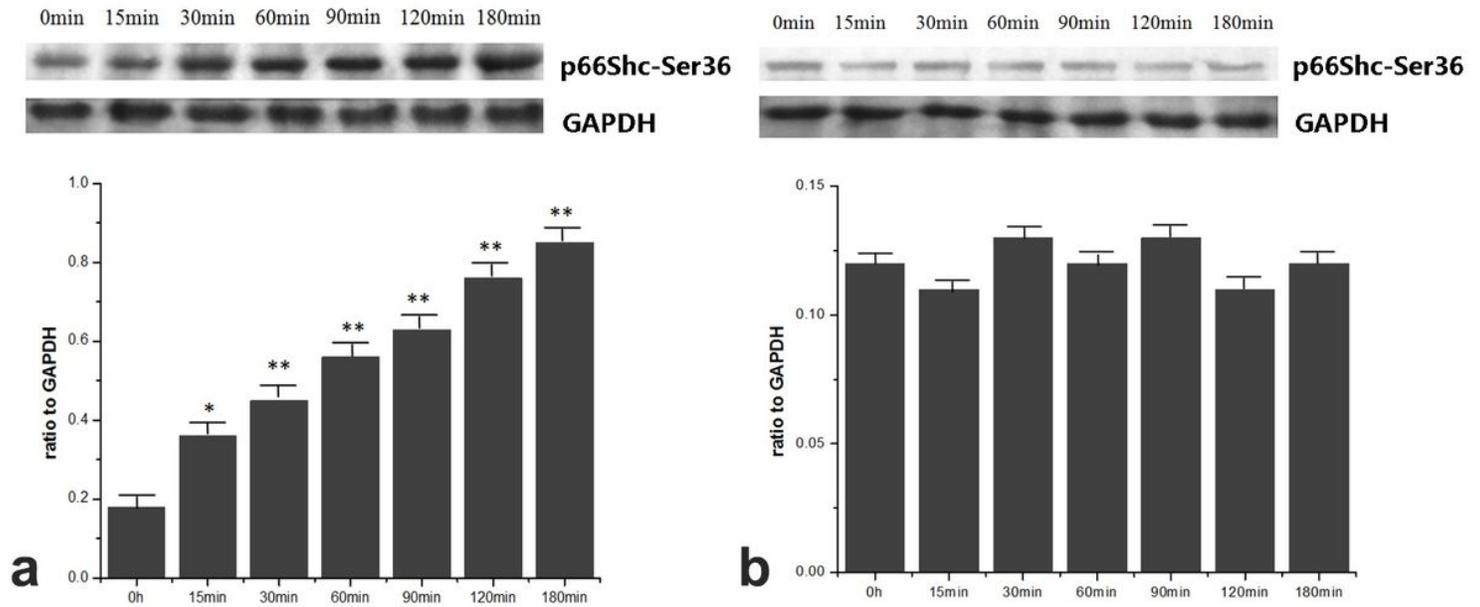


Figure 4

(a) The phosphorylation of p66Shc Ser36 in R28 cells at different time points was detected by western blot. The results showed that the phosphorylation of p66Shc Ser36 was significantly increased after 15 minutes of glucose treatment, and with the prolongation of glucose exposure time, the protein level increased gradually, and there was a significant difference among the groups. (b) The level of phosphorylated Ser36 p66Shc protein in R28 cells stimulated with isotonic mannitol (30 mM) did not change significantly with the prolongation of treatment time. (*: compared with group 0h, $P < 0.05$; **: compared with group 0h, $P < 0.01$).

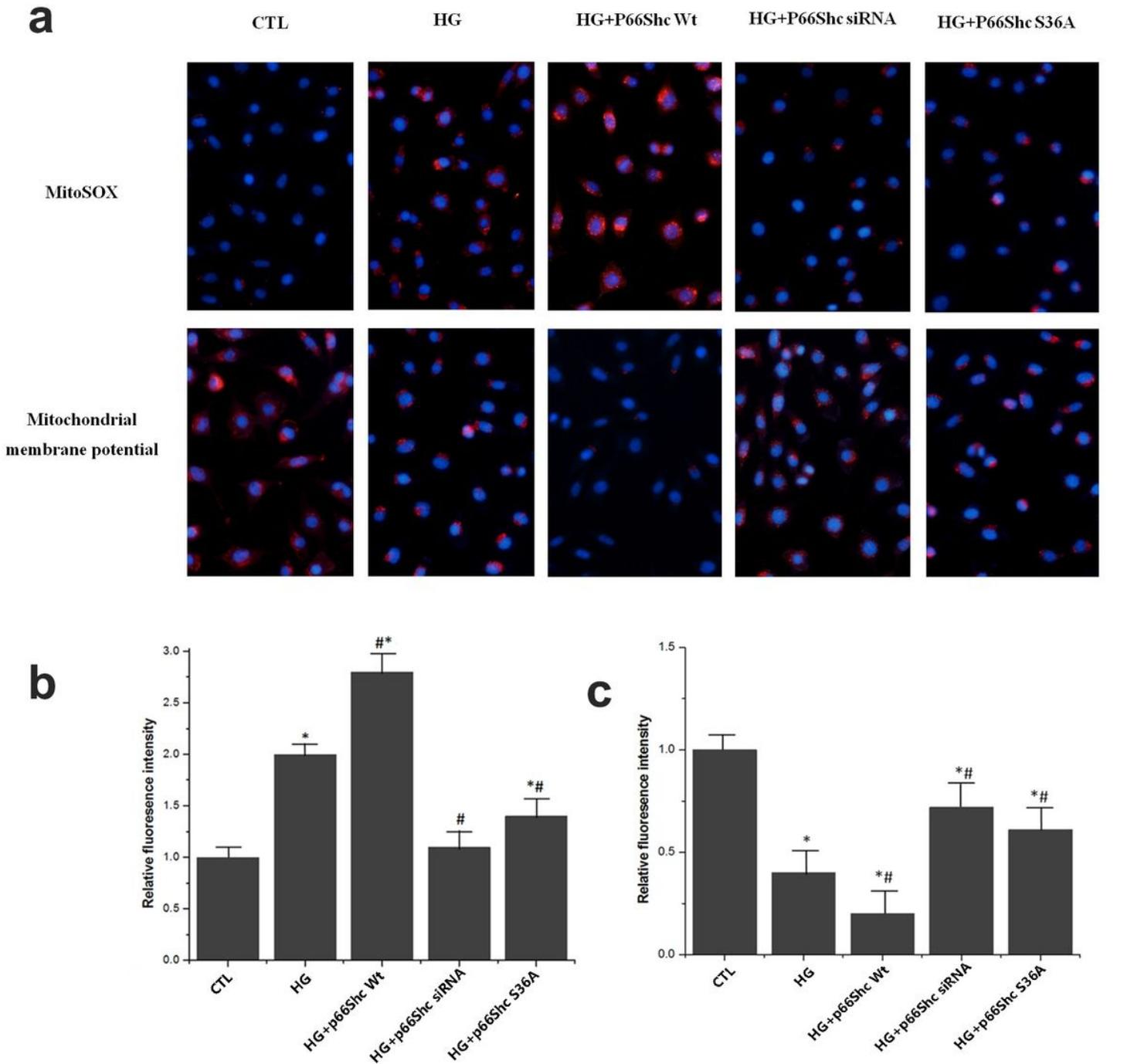


Figure 5

(a) Confocal microscopy images of cells subjected to MitoSOX and mitochondrial membrane potential levels. By MitoSOX staining, the nucleus showed blue fluorescence and ROS showed red fluorescence. After 24 hours of glucose stimulation, the level of red fluorescence was increased in the HG group, and was further increased in the HG p66Shc Wt group. In contrast, compared with the HG group, the red fluorescence of HG p66Shc siRNA group and HG p66Shc S36A group decreased. Detection of the mitochondrial membrane potential, the mitochondrial membrane potential was red and the nucleus was blue. The red fluorescence expression was decreased in the HG group, and was further decreased in the

HG p66Shc Wt group. The red fluorescence expression of the HG p66Shc siRNA group and the HG p66Shc S36A group recovered partially. (b) the bar graph represents a quantification of MitoSOX analyses of cells. (c) the bar graph represents a quantification of the mitochondrial membrane potential analyses of cells. (*: compared with the normal control group, $P < 0.05$; #: compared with the HG group, $P < 0.05$).

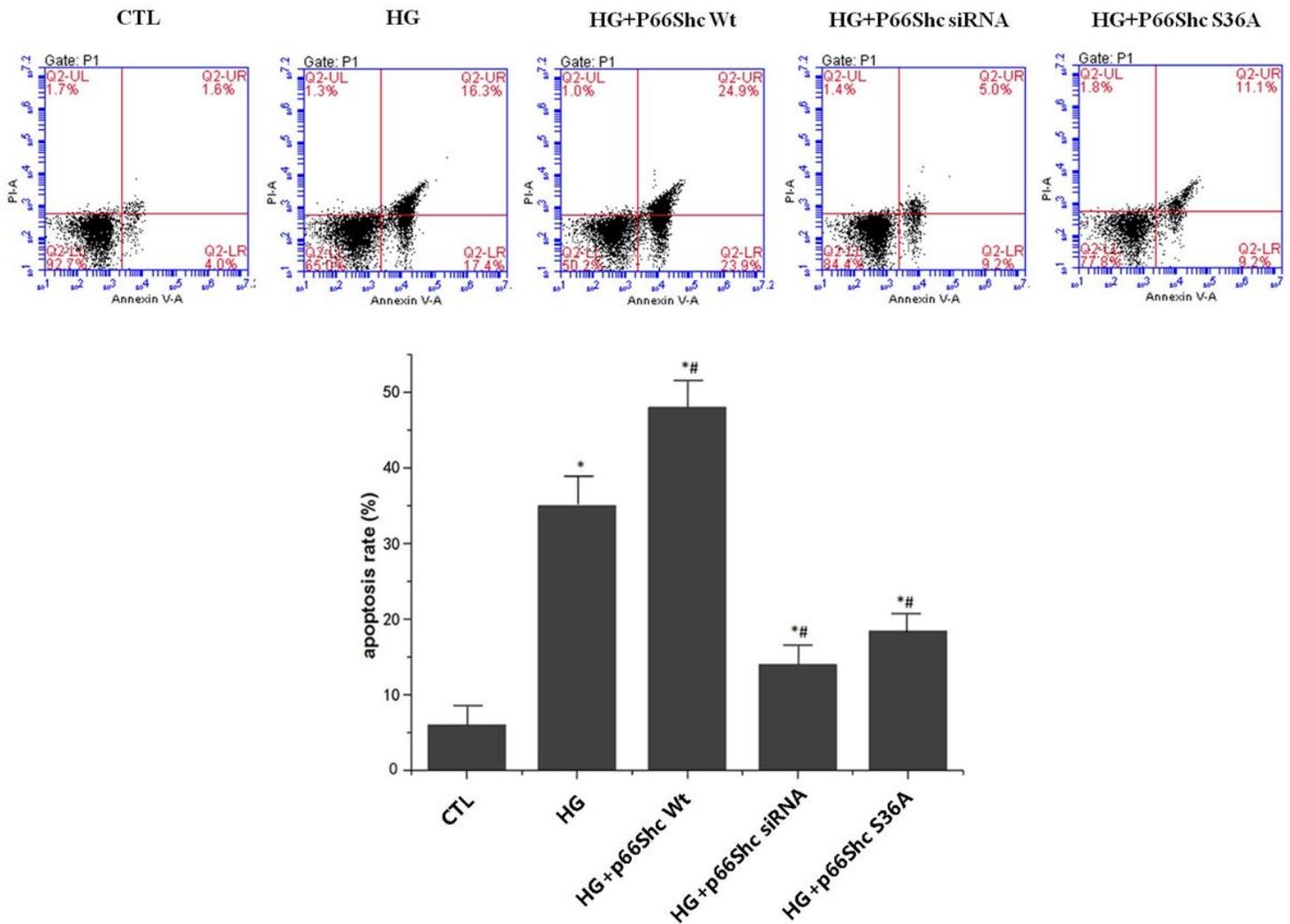


Figure 6

Flow cytometry was used to detect apoptosis rate of each group. Compared with the normal control group, the apoptosis rate was significantly higher in the HG group. Among all the groups, the rate of apoptosis was the highest in the HG p66Shc Wt group. However, compared with the HG group, the apoptosis rate in the HG p66Shc siRNA group and the HG p66Shc S36A group were significantly decreased. (*: compared with the normal control group, $P < 0.05$; #: compared with the HG group, $P < 0.05$).

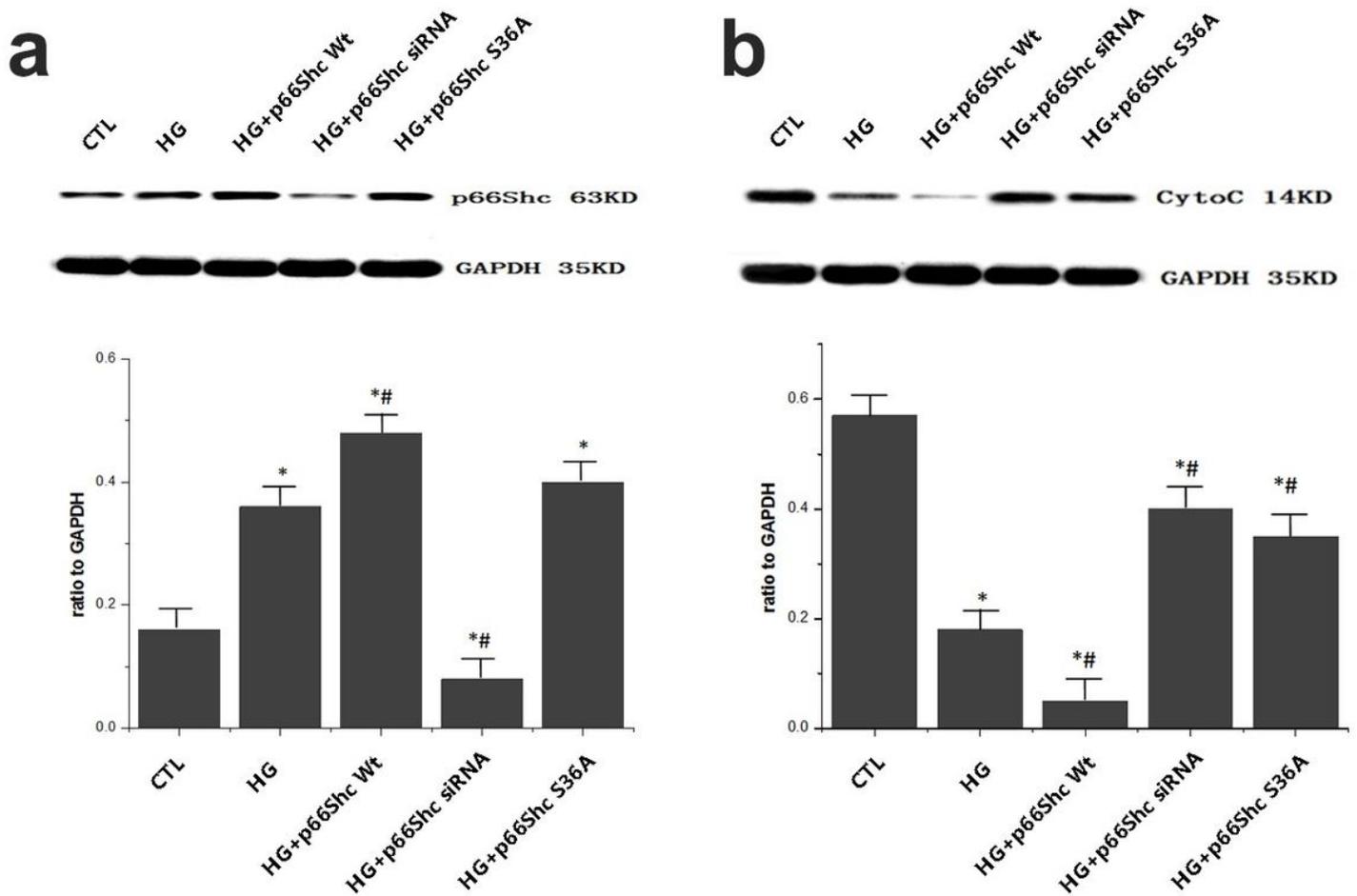


Figure 7

(a) Western blot was used to detect the expression of p66Shc in mitochondria. The expression of p66Shc in the mitochondria of the HG group was significantly higher than that of the normal group, and was the highest in the HG p66Shc Wt group. However, compared with the HG group, the expression of p66Shc in the HG p66Shc siRNA group was significantly decreased. There was no significant difference in the expression of p66Shc in mitochondria between the HG p66Shc S36A group and the HG group. (b) Western blot was used to detect the expression of cytochrome C in mitochondria. The expression of cytochrome C in mitochondria in the HG group was significantly lower than that in normal group, and was the lowest in the HG p66Shc Wt group. However, compared with the high glucose group, the expression of cytochrome C in mitochondria in the HG p66Shc siRNA group and the HG p66Shc S36A group were both significantly increased. (*: compared with the normal control group, $P < 0.05$; #: compared with the HG group, $P < 0.05$).

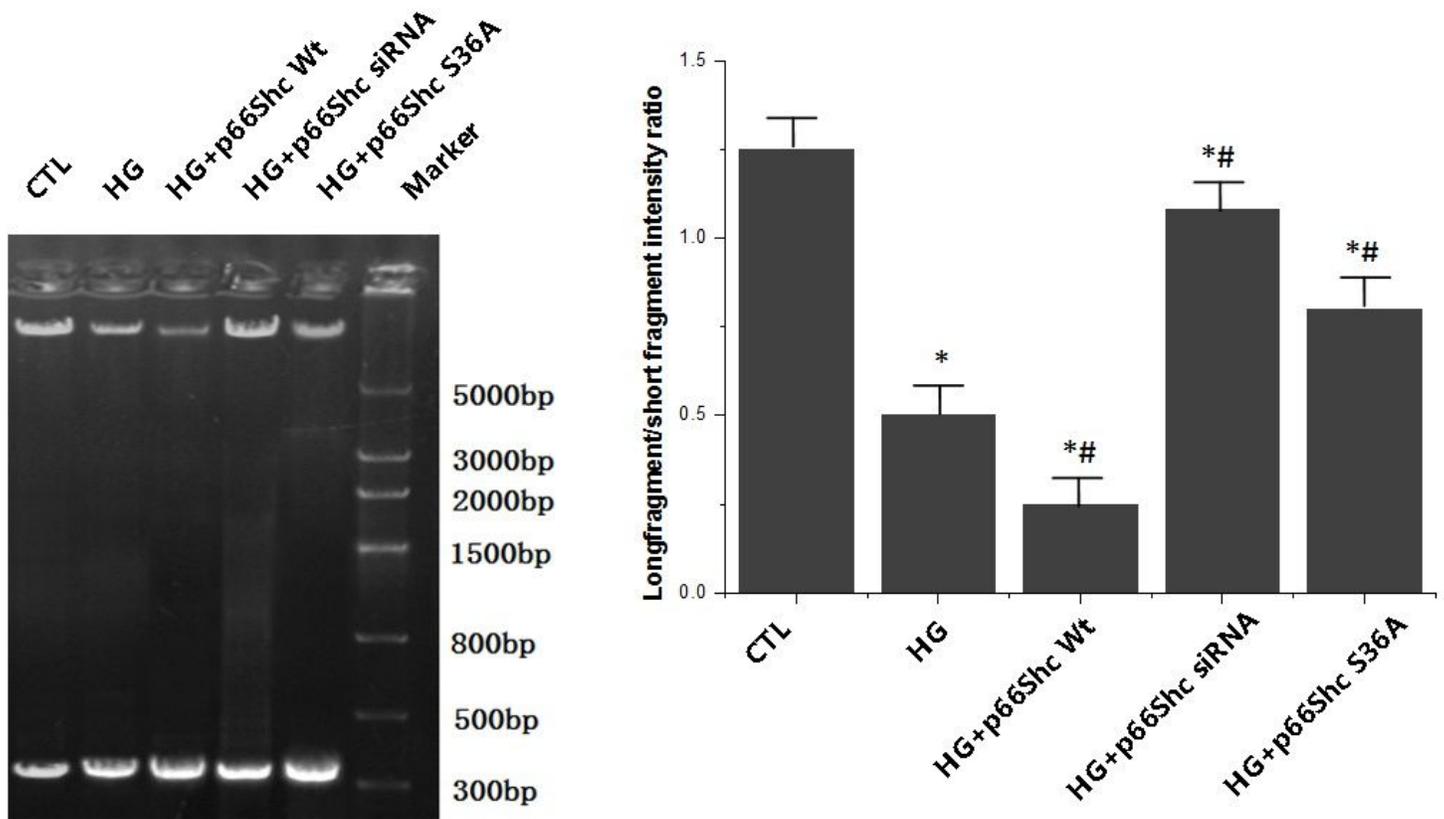


Figure 8

Compared with the normal group, the proportion of long fragments of mitochondrial DNA in the HG group was decreased significantly. The proportion of long fragments of mitochondrial DNA decreased further in the HG p66Shc Wt group. However, this was attenuated in the HG p66Shc siRNA and HG p66Shc S36A groups. (*: compared with the normal control group, $P < 0.05$; #: compared with the HG group, $P < 0.05$).