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Original Article

Notch signaling protects retina from nuclear factor-κB- and poly-ADP-ribose-polymerase-mediated apoptosis under high-glucose stimulation

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Proliferative diabetic retinopathy, the primary cause of vision loss in adults, is one of serious microvascular complications caused by diabetes. Both poly-ADP-ribose-polymerase (PARP) and nuclear factor (NF)-κB signaling are involved in the injury process. Injury activates PARP, which in turn potentiates NF-κB activation and causes cell apoptosis. Like the NF-κB pathway, Notch1 signaling plays a key role in the regulation of cell proliferation, differentiation, and apoptosis. However, the connections between these signaling pathways are not well understood. In this study, we used both streptozotocin (STZ)-induced diabetic mice and human retinal vascular endothelial cells (HRVECs) cultured in high glucose to detect these relationships. We found that apoptosis was increased in both STZ-induced diabetic mice and high-glucose-treated HRVECs, which was due to increased activation of PARP, cleaved caspase3, and reduced expression of Notch1 and p-Akt. The results of Notch1 overexpression and knockdown indicated that Notch1 signaling participated in the interaction of PARP and p50, and inhibited PARP- and p50-mediated apoptosis directly. These phenomena could be blocked by pretreatment with the PI3K inhibitor wortmannin via reducing p-Akt levels. Thus, our study demonstrated that Notch1 signaling protects cells from PARP- and NF-κB-induced apoptosis under high glucose through the activation of Akt.

Keywords diabetes; retina; NF-κB; Notch signaling; apoptosis

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Introduction

Diabetic retinopathy (DR), initially characterized by retinal ischemia, can progress to a proliferative stage involving neovascularization of the retinal vasculature and neural retina, leading to visual disturbance and eventually to blindness [1,2]. The known pathogenetic mechanisms of DR are focused on oxidative stress, increased aldose reductase activity, activation of protein kinase C, non-enzymatic glycation, and advanced glycation end products, which have been proved in experimental studies using animal models of early and advanced DR [3–5]. In DR, both nuclear factor (NF)-κB and poly-ADP-ribose-polymerase (PARP) are downstream mediators of oxidative injury [6–9]. PARP-1 plays a role in DNA repair and regulates the expression of various proteins at the transcriptional level, including many inflammatory mediators that are also regulated by the transcription factor NF-κB, which also controls the expression of several anti-apoptotic factors. NF-κB1 (p50) and RelA (p65) constitute a heterodimer, the major form of NF-κB, each of which contains an N-terminal Rel homology domain [10]. However, p50 subunits cannot directly regulate the transcription of target genes due to the lack of C-terminal transcription activation domain [11]. Thus, p50 often exerts it transcriptional activity through interacting with other factors [11,12]. PARP has been shown to cause NF-κB activation in diabetes [13]. PARP can be activated by oxidative stress-induced DNA single-strand breaks, which in turn potentiates NF-κB activation, resulting in higher expression of NF-κB-dependent genes and leads to a positive feedback loop of diabetic vascular injury [14]. Notch signaling activated by its ligands also plays key roles in regulating proliferation, differentiation, and apoptosis. Delta-like ligand 4 (DLL4) is one ligand of the Notch receptor and is largely restricted to the vascular endothelium. In the vascular lesion, DLL4 is a key ligand for Notch signaling activation [15]. Emerging evidences have supported a functional interplay between Notch and NF-κB signaling pathways. Notch can interact with NF-κB and modulate NF-κB-dependent gene transcription [16]. The activation of protein kinase B (Akt) also can be regulated by oxidative stress, which activates I-kappa-B kinase (IKK) [17]. Notch, phosphatidylinositol 3-kinase (PI3K)/Akt, and NF-κB signaling pathways interact through complex molecular

networks in developing, adult, and neoplastic tissues [18,19]. It has also been reported that Notch1 inhibits drug-induced or p53-mediated apoptosis through the PI3K/Akt pathway in neoplastic tissues [20,21]. Although the interaction between PARP and NF-κB signaling in vascular diseases is widely appreciated, little is known about how the Notch signaling pathway is involved in the regulation of NF-κB and PARP in DR. Here, we used streptozotocin (STZ)-induced diabetic mice and high-glucose-cultured human retinal vascular endothelial cells (HRVECs) to investigate the interaction between PARP and NF-κB, and how Notch-Akt pathway regulates PARP and NF-κB signaling. Our study showed that PARP interacted with NF-κB, activated caspase3, and eventually led to cell apoptosis under high-glucose stimulation. These findings indicated that PARP promotes cell apoptosis through the interaction with NF-κB, and Notch signaling protects cells from PARP- and NF-κB-induced apoptosis under high-glucose conditions through Akt activation.

Materials and Methods

Antibodies

Anti-p-Akt, anti-T-Akt, anti-cleaved PARP, and anti-cleaved caspase3 antibodies were from Cell Signaling Technology (Beverly, USA); anti-DLL4 antibody was from R&D Systems (Minneapolis, USA); anti-flag antibody were obtained from Sigma (St Louis, USA); anti-Notch1, anti-p50, and horseradish peroxidase (HRP)-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, USA.).

Diabetic mouse model

To induce diabetes, 8–10-week-old C57BL/6 mice were injected intraperitoneally (i.p.) with STZ (150 mg/kg, Sigma). STZ was prepared by dissolving in citrate buffer (pH 4.5) immediately before administration. Control mice were injected with buffer only. Mice with blood glucose higher than P400 mg/dl 6 weeks after STZ treatment were used as diabetic animals.

Cell culture and treatment

HRVECs (ScienCell Research Laboratories, Carlsbad, USA) were cultured in low-glucose Dulbecco's modified Eagle's media (Invitrogen, Carlsbad, USA) containing 10% (v/v) fetal bovine serum (Gibco, Burlington, Canada), 1% (v/v) penicillin, 2 mM glutamine, and non-essential amino acids at 37°C under humidified conditions with 5% CO_2. Cells from passages 3–7 were used in all experiments. Cells were incubated at the indicated glucose concentrations (10, 20, and 30 mM) for 4 days, respectively. All cell medium were changed with fresh medium every 48 h.

Plasmid construction

The human full-length *Notch1*, *PARP*, *DLL4*, and *p50* cDNA were obtained from HEK293T cells by reverse transcriptase-polymerase chain reaction (RT-PCR) using M-MLV Reverse Transcriptase from Promega (Madison, USA), and confirmed by DNA sequencing. Primer sequences used for RT-PCR in this study were as follows: *Notch1*: forward 5'-GAATTCCCGCCGCTCCTGGCGCCC-3' and reverse 5'- AAGCTTTTACTTGAAGGCCTCCGG-3'; *p50*: forward 5'- AAGCTTGCAGAAG ATGATCCATATT-3' and reverse 5'-AGATCTCTAAATTTTGCCTTCTAGA-3'; *PARP*: forward 5'-AGATCTGCGG AGTCTTCGGATA AGCTC-3' and reverse 5'-GAATTCTTACCACAGGGA GGTCTTAAA-3'; *DLL4*: forward 5'-GGATCCCATAT GGCGGCAGCGTCCCGGAGCGCCTC-3' and reverse 5'-GAATTCTTATACCTCCGTGGCAATGACACATTCA-TTC-3' [22].

The cDNAs of *Notch1* and *DLL4* were subcloned into pCMV-Script vector (Merck, Darmstadt, Germany) to construct the recombinant plasmids pCMV-Script-Notch1 and pCMV-Script-DLL4. The cDNA of *p50* was subcloned into the pFlag-CMV vector (Sigma) to construct the recombinant plasmid pFlag-CMV-p50. The cDNA of *PARP* was subcloned into the pEGFP-C1 vector (Clontech, Mountain View, USA) to construct recombinant plasmid pEGFP-C1-PARP.

siRNA and cDNA transfection

HRVECs were seeded in 60 mm plates, cultured for 24 h, and then were transfected with siRNA targeting *Notch1* (Santa Cruz), pCMV-Script-Notch1, pFlag-CMV-p50, pCMV-Script-DLL4, or pEGFP-C1-PARP, using LipofectamineTM 2000 (Invitrogen, Paisley, UK) according to the manufacturer's protocol. DMEM containing 30 mM glucose was added after transfection 4 h. At 48 h post-transfection, cells were treated with or without 1 μM wortmannin (Sigma) for 30 min and collected for further analysis.

Real-time PCR

Total RNA was extracted from cells by using TRIzol Reagent (Invitrogen) and was reverse transcribed using M-MLV Reverse Transcriptase. Real-time PCR was performed using an ABI 7500 fast sequence detection system (Applied Biosystems, Foster City, USA) with SYBR green fluorescent label. The primer sequences used in the quantitative PCR assays for human *Notch1* were as follows: *Notch1* forward primer, 5'-GAGGCGTGGCAGACTAT GC-3'; reverse primer, 5'-CTTGTACTCCGTCAGCGT GA-3'. *β-actin* was used as the control. The primers were as follows: forward, 5'-CAGCCATGTACGTTGCTATCC AGG-3'; reverse, 5'-AGGTCCAGACGCAGGATGGC ATG-3'. Ten microliters of samples contained 1 μl Power SYBR Green PCR Master Mix (Applied Biosystems),

5 pmol of each primer, and 0.25 μl of the RT reaction were run in triplicate in optically clear 96-well plates (Corning, New York, USA). Cycling parameters were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, and an extension step at 72°C for 30 s. *β-actin* was used in each sample as an internal reference to standardize the results by eliminating variations in mRNA quantity.

Western blot analysis

Retinal tissues were quickly dissected on an ice-cold plate, frozen in liquid nitrogen, and stored at −80°C. Each retina was homogenized in 10 volumes of sample buffer [20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.02% sodium azide, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 5 g/ml leupeptin]. Cell lysates were prepared using the same buffer as the retinal tissues. Equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, USA). The membranes were blocked with 5% non-fat milk at room temperature for 2 h, and then incubated with the appropriate primary antibody (1:1000) overnight at 4°C. After being washed with Tris-buffered saline Tween-20 (TBST) three times, the membranes were then incubated with HRP-conjugated secondary antibody (1:5000) at room temperature for 1 h. The bands were visualized with an enhanced chemiluminescence reagent (Amersham Biosciences, Buckinghamshire, England). Band intensities were quantified by Quantity One software (Bio-Rad, Hercules, USA).

Immunohistochemistry

Normal and diabetic mice were sacrificed. The eyes were enucleated, fixed overnight with 4% paraformaldehyde, processed, embedded in paraffin blocks, and cut into 4 μm sections. After being blocked with non-specific antibody 2% normal goat serum for 1 h, sections were incubated with specific antibodies of anti-Notch1 (1:100) and anti-p50 (1:100) overnight at 4°C. HRP-conjugated secondary antibodies were applied (1:5000) for 2 h at room temperature. Diaminobenzidine was used to develop color.

Confocal microscopy

Cells were grown on microscopic coverslips until they reached ~80% confluence. Then cells were washed twice with PBS, fixed in 4% paraformaldehyde at room temperature for 20 min, permeabilized with PBS containing 0.3% Triton X-100 (PBST) for 15 min, and blocked in 5% goat serum for 1 h. The cells were incubated with anti-flag antibody (1:100) at room temperature for 2 h, and then incubated with rhodamine-conjugated secondary antibody. Slides were mounted in Vectashield fluorescence mounting medium with DAPI (Sigma) for nuclear staining and visualized under a confocal laser scanning microscope (Olympus, Center Valley, USA).

Co-immunoprecipitation

Cells were harvested in lysis buffer [20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.02% sodium azide, 1 mM PMSF, and 5 g/ml leupeptin]. Lysates were clarified by centrifugation at 12,000 *g* for 30 min at 4°C. The supernatant was precleared with protein A-Sepharose (Santa Cruz) for 1 h at 4°C, followed by incubation with the indicated primary antibodies under gentle rocking at 4°C overnight. Protein A-Sepharose was added, and the mixture was incubated for an additional 1 h at 4°C. The precipitates were washed with lysis buffer and analyzed by SDS-PAGE and immunoblotting.

Apoptosis assay

Cells were harvested and prepared as single-cell suspensions by mechanical blowing with PBS, washed twice with cold PBS. Then cells were fixed with 70% alcohol at 4°C for 24 h and stained with propidium iodide. Cell apoptosis was detected using flow cytometry. Three independent experiments were performed.

Results

Notch1 expression is reduced significantly in diabetic mouse retina in association with decreased levels of DLL4 and p-Akt

To study the expression of Notch1 and p50 protein in diabetic mouse retina, immunohistochemical analysis was performed on retina specimens from normal and STZ-induced diabetic mouse eyes. The results revealed that the expression levels of precursor, transmembrane, and intracellular Notch1 were significantly reduced in STZ-induced diabetic mouse retina compared with the normal group [**Fig. 1(A)**]. However, the expression level of p50 did not show an obvious change. Similar results were observed in immunoblots. Accordingly, the expression levels of DLL4, the receptor of Notch1, and p-Akt revealed a significant decrease in STZ-induced diabetic mouse retina. Additionally, we noticed that two apoptosis indicators, the cleaved PARP and cleaved caspase3, were activated in STZ-induced diabetic mouse retina [**Fig. 1(B)**]. These results indicated that more mouse retina cells underwent apoptosis in the STZ-induced diabetic mouse model than in the normal mouse, which may be mediated by the Notch1-Akt pathway.

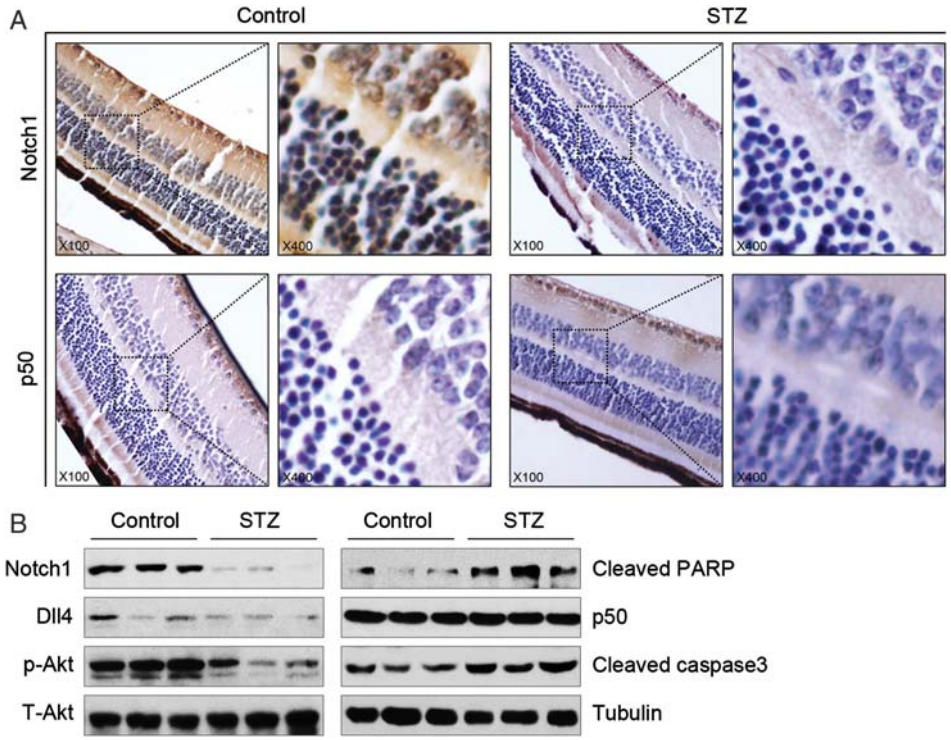

Figure 1 Notch1 expression is reduced significantly in diabetic mouse retina in association with decreased levels of DLL4 and p-Akt (A) Paraffin sections of tissues from eyes of non-diabetic control (control) and STZ-induced diabetic mice (STZ) were stained with anti-Notch1 and anti-p50 antibodies. (B) The retinal tissues lysates were subject to western blot analysis, probed with anti-Notch1, anti-p50, anti-DLL4, anti-cleaved PARP, anti-p-Akt, anti-T-Akt, and anti-cleaved caspase3 antibodies. Tubulin was used as the loading control.

Notch1-Akt signaling is involved in high-level glucose-induced apoptosis *in vitro*

To further investigate the relationship between Notch1-Akt and apoptosis, we treated HRVECs with different concentrations of glucose. Immunoblotting indicated that glucose stimulation induced increased expression of cleaved PARP and cleaved caspase3, which suggested that glucose could induce apoptosis of HRVECs. Notably, under high concentration of glucose (30 mM), the expression levels of both active Notch1 (80 kDa) and p-Akt were decreased significantly. Expression did not change in a significant way with low concentrations of glucose (10, 20 mM) stimulation [**Fig. 2(A)**].

When Notch1 and its receptor DLL4 were overexpressed in cells exposed to high glucose, the percentage of apoptotic cells was dramatically reduced. This decrease can be reversed by wortmannin, a PI3K inhibitor that can reduce p-Akt levels [**Fig. 2(B)**]. These results suggested that Notch1-Akt signaling is involved in high-glucose-induced apoptosis *in vitro*.

PARP interacts with p50 in endothelial cells with high-glucose stimulation

The subcellular distributions of PARP and p50 in normal and high-glucose-treated HRVECs were also examined by confocal immunofluorescence. The results revealed that PARP and p50 were localized in both the nucleus and perinuclear areas in normal endothelial cells, but they were mainly localized in the nucleus after high-level glucose stimulation, more significantly for p50. Overexpressed Notch1 caused p50 to relocate to perinuclear areas, while PARP did not show obvious changes in localization [**Fig. 3(A)**]. These results suggested that PARP might interact with p50 in high-glucose-treated HRVECs.

To further identify whether there is an interaction between PARP and p50, we performed immunoprecipitation with the extracts of normal and high-glucose-induced HRVECs. As shown in **Fig. 3(B)**, PARP interacted with p50 in endothelial cells, and the interaction could be enhanced by high-level glucose stimulation. Immunoprecipitation analysis was also performed to detect the interaction between PARP and p65, another important subunit of NF-κB, and no significant difference of PARP/p65 interaction was detected between the high-glucose-treated group and the control group.

Notch1 inhibits high-glucose-induced apoptosis

The whole protein extracts from the endothelial cells subjected to different treatments were analyzed using western blot. As shown in **Fig. 2**, the protein levels of cleaved PARP and cleaved caspase3 under high-glucose condition were obviously higher than those in normal cells. When Notch1 was overexpressed in the normal cultured cells, there were

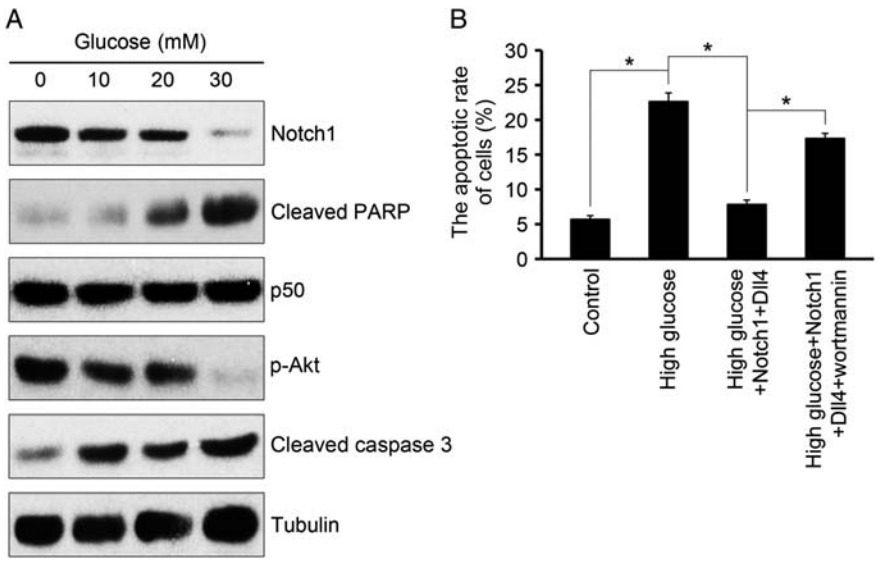

Figure 2 Notch1-Akt signaling is involved in high-level glucose-induced apoptosis *in vitro* (A) HRVECs were incubated in the indicated concentrations of glucose for 4 days. Then the whole-cell extracts were immunoblotted with antibodies against Notch1, cleaved PARP, p-Akt, p50, and cleaved caspase3. Tubulin was used as the loading control. (B) Overexpressed Notch1 and its receptor DLL4 in cells reduced high-glucose-induced apoptosis, which could be rescued by adding wortmannin, an inhibitor of PI3K. $*P < 0.05$ vs. control. Calculated from three independent experiments.

no obvious differences [**Fig. 4(A,B)**], indicating that notch1 did not affect normal cells. When Notch1 was introduced to the cultured cells exposed to high-glucose alone, the expression levels of cleaved PARP and cleaved caspase3 were significantly reduced [**Fig. 4(A,B)**]. If both Notch1 and DLL4 were introduced into high-glucose-stimulated cells, the reduction of cleaved caspase3 was more significant compared with that in cells overexpressing Notch1 alone [**Fig. 4(B)**]. An opposite response of p-Akt was also observed [**Fig. 4(A)**], which was consistent with the previous results [**Figs. 1(B), Fig. 2(A)**].

Notch1-Akt signaling regulates PARP and p50 interaction

To further study whether Notch1 could affect the interaction between PARP and p50, we performed co-immunoprecipitation in cultured endothelial cells under the indicated treatment conditions. Immunoblotting results suggested that high-glucose stimulation could increase the interaction between PARP and p50. However, when we introduced Notch1 and DLL4 to high-glucose-treated cells, the interaction between PARP and p50 was significantly impaired. This reduction could be blocked by wortmannin [**Fig. 5(A)**]. Knockdown of Notch1 could also rescue it [**Fig. 5(B)**]. These results demonstrated that Notch1-Akt signaling regulates the PARP/p50 interaction.

Notch1-Akt signaling has an inhibitory effect in high-glucose-induced apoptosis

We have demonstrated that Notch1 can inhibit cell apoptosis in high-glucose-cultured endothelial cells. We then investigated how p-Akt participates in this process. We found that when wortmannin was added to the high-glucose-cultured endothelial cells with Notch1 and DLL4 overexpression, the cleavage of PARP and caspase3 was increased significantly again [**Fig. 5(C)**]. These results indicated that the inhibitory effect of Notch1 in high-glucose-induced apoptosis is at least partially mediated by p-Akt. Furthermore, in high-glucose-cultured cells, overexpressed DLL4 alone reduced the expression of cleaved PARP and cleaved caspase3, whereas p-Akt expression was increased under this condition. Using the RNAi technique, Notch1 was inhibited in HRVECs [**Fig. 5(E,F)**]. Knockdown of Notch1 by siRNA increased PARP and caspase3 cleavage accompanied with decreased p-Akt. Co-transfection with both DLL4 and siNotch1 in high-glucose-cultured cells did not have significant effect compared with the cells transfected with siNotch1 alone [**Fig. 5(D)**]. Taken together, the above findings indicated that the increased cleavage of PARP and caspase3 induced by high-glucose stimulation is directly mediated by Notch1-Akt signaling.

Discussion

NF-κB is a key transcription factor involved in cell survival, cancer, and inflammation. PARP, an abundant nuclear enzyme involved in DNA repair, has been shown to act as a co-activator in NF-κB-mediated transcription [14]. Some studies have indicated that PARP inhibitor prevents the diabetes-induced elevation in circulating nitrite levels in STZ-induced diabetes. It has been reported that PARP

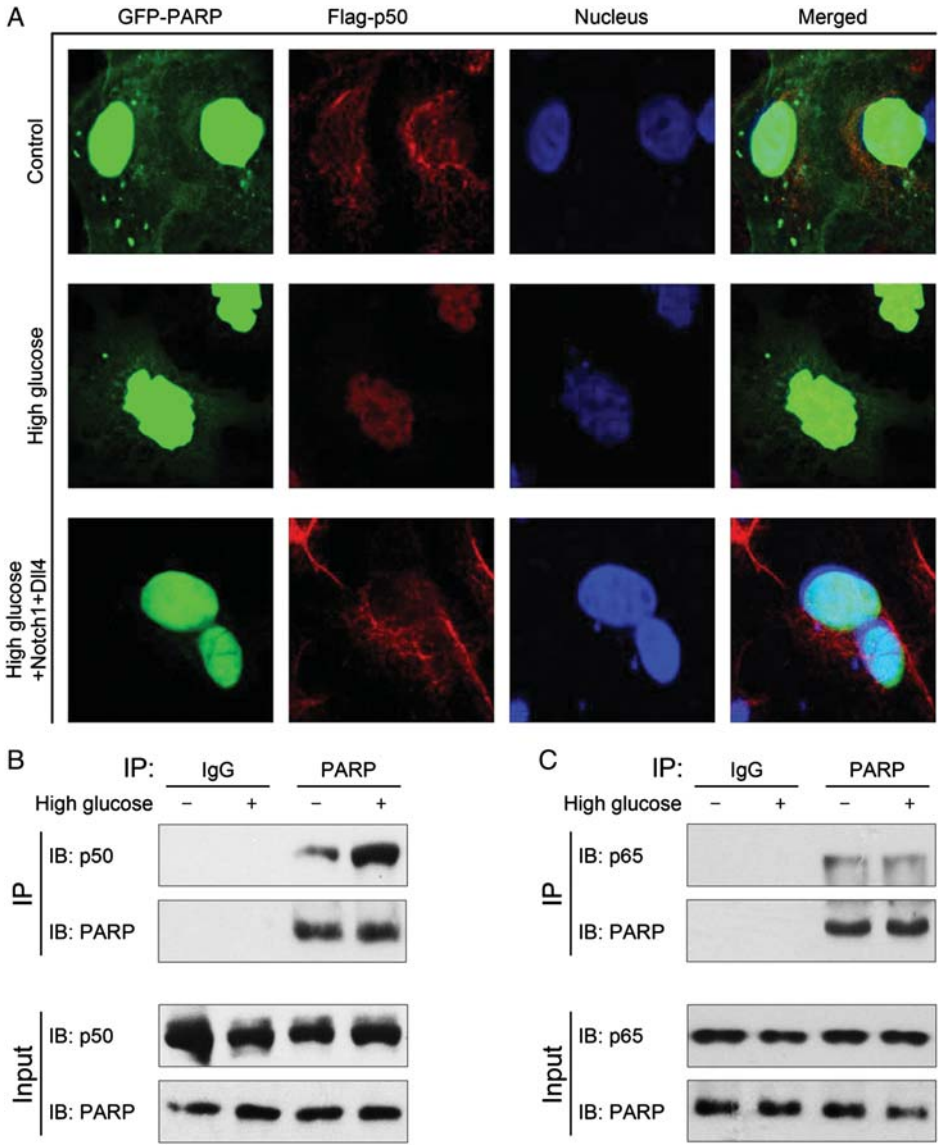

Figure 3 PARP interacts with p50 in endothelial cells stimulated with high glucose (A) Subcellular distribution of EGFP-PARP (green) and flag-p50 (red) in endothelial cells under the indicated treatments. The control group and the high-glucose group were cotransfected with pFlag-CMV-p50 and pEGFP-C1-PARP and treated with or without high glucose (30 mM). The high glucose+Notch1+DLL4 group was cotransfected with pFlag-CMV-p50, pEGFP-C1-PARP, pCMV-Script-Notch1, and pCMV-Script-DLL4 and treated with 30 mM glucose. The cells were fixed and stained for Flag-p50 (red, rhodamine) and nuclei (blue, DAPI). The EGFP tagged PARP are seen in green. (B) PARP was immunoprecipitated from lysates of cells treated with or without high glucose (30 mM). The precipitates were immunoblotted for p50 and PARP. IgG was used as the negative control. (C) PARP was immunoprecipitated from lysates of cells treated with or without high glucose (30 mM). The precipitates were immunoblotted for p65 and PARP. IgG was used as the negative control.

deficiency suppresses NF-κB activation in cultured endothelial cells under high-glucose stimulation [9]. Furthermore, it has been shown that NF-κB is regulated by PARP in DR [6]. In our experiments, we found that cleaved PARP and caspase3 levels were increased in the retina of STZ-induced diabetic mice and high-glucose-induced HRVECs, and cell apoptosis could be induced by glucose in a dose-dependent manner (**Figs. 1** and **2**). Proteolytic cleavage of PARP-1 within module B by activated caspase3, which generates an amino-terminal 25-kDa and a carboxy-terminal 89-kDa fragment, has been reported during the execution phase of apoptosis in a wide range of organisms [23]. Our results revealed that PARP played a role in the induction of cell apoptosis, and therefore in the pathogenesis of DR. NF-κB activation can also induce pro-apoptotic effects in the pathogenesis of the endothelial dysfunction associated with diabetes [15]. Some reports have indicated that NF-κB expression is increased in parallel with inner retinal injury following retinal ischemia in rats, suggesting a possible pro-apoptotic role for NF-κB [8]. To study how PARP and NF-κB are involved in the process of DR pathogenesis, we performed co-immunoprecipitation on normal

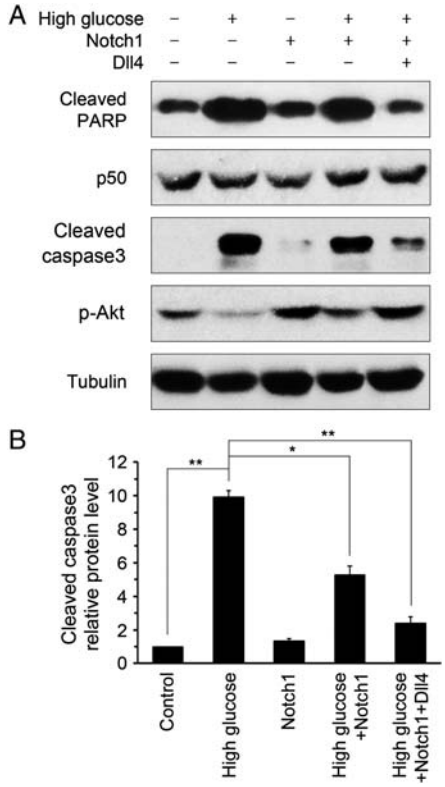

Figure 4 Notch1 inhibits high-glucose-induced apoptosis (A) HRVECs treated with or without high glucose (30 mM) were transfected with the indicated plasmids. The whole-cell extracts were immunoblotted with antibodies against cleaved PARP, p-Akt, p50, and cleaved caspase3. Tubulin was used as the loading control. (B) The quantitative analysis was performed for the relative levels of cleaved caspase3 above. $*P < 0.05$ and $**P < 0.01$.

endothelial cells and high-glucose-induced cells to detect the protein interaction between PARP and NF-κB. We found that p50 could interact with PARP, the levels of which were increased in high-glucose-induced cells [**Fig. 3(B)**]. Confocal microscopy revealed that PARP and NF-κB were translocated into the nucleus from perinuclear areas in HRVECs exposed to high glucose [**Fig. 3(A)**]. All these results suggested that PARP might activate NF-κB by entering the nucleus, where it combines with NF-κB to activate caspase3 and finally leads to cell apoptosis related to DR.

Notch1 signaling is highly conserved and can regulate cell growth, proliferation, and differentiation during vascular development. The Notch family of receptors are large single-pass type I transmembrane proteins represented by four members, Notch1 to Notch4, in mammals. These receptors interact with five ligands (DLL1, 3, 4, Jagged1, 2) [24]. Notch1 is mainly expressed in vascular tissues, and DLL4 is largely restricted to the vascular endothelium, suggesting that Notch1 and DLL4 play key roles in the development of vasculature and pathological angiogenesis [25,26]. Gene deletion of Notch1 and DLL4 results in embryonic lethality associated with vascular remodeling deficiency. It has been reported that both the size of the residual avascular area and the development of epiretinal neovascular tufts are substantially reduced in DLL4$^{+/lacZ}$ mice compared with their wild-type littermates in the Oxygen-Induced Retinopathy model. These phenomena indicate that DLL4 can act as a negative regulator of capillary sprouting during pathologic states, as well as during normal development [27]. Some researchers believe that the loss of Notch1 or haploinsufficiency of the Notch ligand DLL4 leads to impaired vascular development and enhanced apoptosis of endothelial cells in the placenta and yolk sac [28–30]. In our study, we found that both notch1 and DLL4 expression levels were significantly reduced in STZ-induced diabetic retina and high-glucose-cultured HRVECs (**Figs. 1** and **2**). We also found that the cleaved PARP and caspase3 expression and the percentage of cell apoptosis induced by high glucose were reduced obviously by overexpressed Notch1 and DLL4 (**Figs. 2** and **4**). These results suggested that Notch1 might have the opposite role of PARP in high-glucose-cultured cells. To further verify that Notch1 protect cells from apoptosis, siNotch1 was designed and transfected into high-glucose-cultured cells. We demonstrated that cleaved PARP expression reduced by DLL4 could be rescued by siNotch1 [**Fig. 5(D)**]. Our results also indicated that Notch1 signaling can regulate the interaction between p50 and PARP [**Fig. 5(A,B)**]. All these results demonstrated that Notch1 signaling plays a key role in protecting cells from apoptosis mediated by PARP and NF-κB.

There is emerging evidence supporting a functional interplay between the Notch and NF-κB signaling pathways. It has also been reported that induction of Notch1 and its ligand DLL4 in arterial endothelium is mediated by PI3K/Akt pathways [31]. Interaction between Notch and Akt signaling has been demonstrated in T lineage cells [32]. One report has demonstrated that the anti-apoptotic effects of Notch are dependent on functional PI3K/Akt-mediated signaling [21]. Akt also activates IKK, leading to the dissociation of the NF-κB/IκB complex and subsequent nuclear translocation of the transcription factor NF-κB [17]. However, few studies have been reported in endothelial cells. In the present study, the relationships among Notch1, Akt, and NF-κB signaling pathways in HRVECs were investigated. We found that p-Akt level was reduced in high-glucose-cultured cells. Wortmannin, a PI3K inhibitor, was able to restore apoptosis in cells overexpessing Notch1 and DLL4. These results indicated that the reduced p-Akt can inhibit the protective effects of Notch1 on cell apoptosis mediated by PARP and NF-κB (**Fig. 2**). However, it also indicated that Notch1/DLL4 signaling could block the interaction between PARP and NF-κB induced by high glucose, and wortmannin could rescue it. Moreover, p-Akt expression was relative to Notch1 and DLL4 [**Fig. 4(A), Fig. 5(C,D)**]. These results suggested that Akt is downstream of Notch1. Notch/Akt signaling can regulate

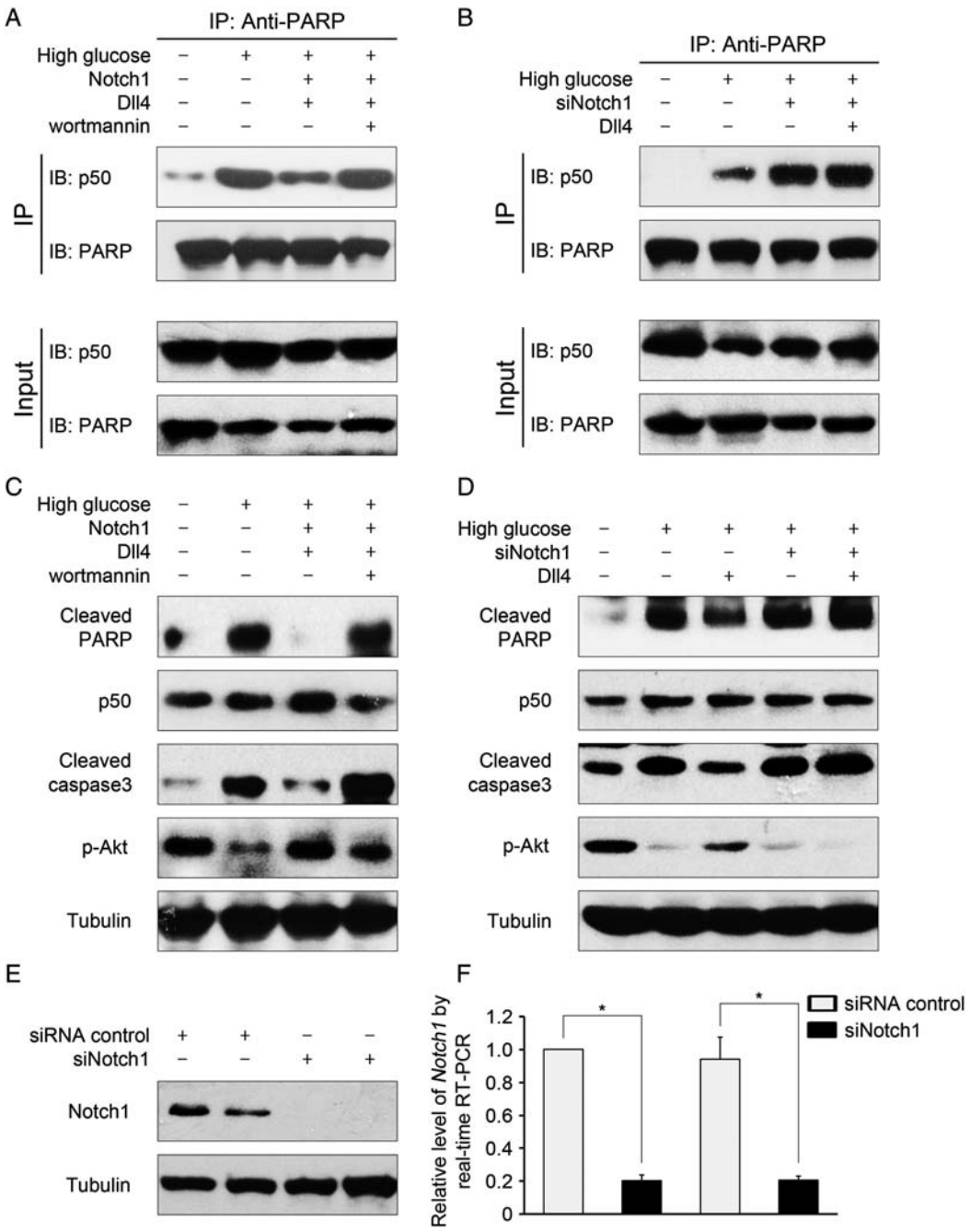

Figure 5 Notch1-Akt signaling regulates PARP and p50 interaction and inhibits cell apoptosis induced by high glucose (A) and (B) Co-immunoprecipitation of p50 in normal or high-glucose-cultured HRVECs transfected with the indicated plasmids or siRNA. Before immunoprecipitation cells were treated with or without wortmannin. The whole-cell extracts were precipitated for PARP and immunoblotted for p50 and PARP. (C) and (D) Western blotting analysis of cleaved PARP, p-Akt, and cleaved caspase3 in endothelial cells with indicated treatments. Tubulin was used as the loading control. (E) siNotch1 inhibited the expression of Notch1 effectively. The HRVECs were transfected with siRNA using LF2000. At 48 h post-transfection, cell lysates were prepared and Western blotting of Notch1 was performed. Tubulin was used as loading control. (F) The expression of *Notch1* mRNA was detected by real-time PCR. After transfection of Notch1 siRNA, the mRNA of *Notch1* was downregulated efficiently. $^*P < 0.01$.

apoptosis through the PARP and NF-κB pathways. The possible mechanisms of apoptosis suppression by Akt [33–36] may include the phosphorylation and inactivation of pro-apoptotic proteins, such as caspase-9 [37], as well as the activation of NF-κB [17].

In conclusion, our studies demonstrated one of the possible mechanisms by which PARP contributes to the development of DR. PARP can be translocated from the cytosol to the nucleus, where it can bind to NF-κB and accelerate the apoptosis of retinal vascular endothelial cells. Together,

our studies suggested that Notch1 signaling protects the endothelial cells from apoptosis mediated by PARP and NF-κB through the Akt pathway.

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