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LABORATORY STUDY

Inhibition to DRP1 translocation can mitigate p38 MAPK-signaling pathway activation in GMC induced by hyperglycemia

LieMei Zhang*, Ling Ji*, XiaoHong Tang, XiaoLei Chen, Zi Li, XuHua Mi, and LiChuan Yang

Division of Nephrology, West China Hospital of Sichuan University, Chengdu, Sichuan, China

Abstract

Diabetic nephropathy (DN) is a serious complication of diabetes with a poorly defined etiology and limited treatment options. Early intervention is a key to preventing the progression of DN. Dynamin-related protein 1 (DRP1) regulates mitochondrial morphology by promoting its fission and is involved in the pathogenesis of numerous diseases. Furthermore, DRP1 is also closely associated with the development of diabetes, but its functional role in DN remains unknown. This study investigated the effect of DRP1 on early stage of DN. DRP1 expression has increased significantly in glomerular mesangial cell (GMC), which is cultivated in high glucose (HG). Ultramicrostructural changes of nephrons, expression of collagen IV and phosph-p38, ROS production, and mitochondrial function were evaluated and, at the same time, were compared with glomerular mesangial cell (GMC) cultured in normal-glucose (NG), mannitol, and a medium with mitochondrial division inhibitor 1 (Midivi-1). Endogenous DRP1 expression increased in DN. Compared to the control groups ofNG and mannitol, overexpression of DRP1 destroyed pathological changes typical of the GMC, like accumulation of extracellular matrix, and an increase in mitochondria division. In addition, Overexpression of DRP1 promoted the activation of p38, the accumulation of ROS, mitochondrial dysfunction, and the synthesis of collagen IV, and all these changes are suppressed by Midivi-1. This study demonstrates that DRP1 overexpression can accelerate pathological changes in the GMC cultured in HG. Further studies are needed to clarify the underlying mechanism of this destructive function.

Keywords

Diabetic nephropathy, DRP1, mitochondria

History

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Introduction

One of the major morbidity and mortality factors confronted by patients with diabetes is an increased risk of developing diabetic nephropathy (DN) that often progresses to end-stage renal disease, 30–40% type 1 diabetes, and 20–30% type 2 diabetes developed to DN after 20–30 years.[1–3] DN is a serious threat to human health, and its current treatments are limited.[4] Early pathology of DN includes mesangial cell proliferation, mesangial extracellular matrix (ECM) accumulation, glomerular basement membrane thickening, and glomerular hypertrophy, which are followed eventually by diffuse nodular glomerulosclerosis.[5] Various mechanisms are implicated in the pathogenesis of DN, including increased aldose reductase activity,[6] enhanced activity of protein kinase C isoforms,[7] and increased formation of advanced glycation end products.[8] However, the development mechanism of DN is complex and has not been completely clarified, making it difficult for early intervention study.

As intrinsic cells of glomerulus, GMCs, which is the main pathological changes of DN, generate ECM, secrete cytokines, and regulate intraglomerular capillary flow, all of which play vital roles in maintaining the organizational structure and physiological function of the kidney.[9,10] Multiple studies have shown that GMC is the main pathological change in DN, and mesangial cell reveals oxidative stress in cascade under high glucose (HG): cell damage, mesangial matrix deposition, and mesangial cell apoptosis, resulting in glomerular dysfunction.[11,12] Mesangial cell apoptosis can further promote the development of DN.[13–15] As DN progresses, ECM, which includes collagen IV, is deposited in the mesangium, causing gradual obliteration of glomerular capillary loops and progressive decline in glomerular filtration.[16]

Mitochondria are the essential organelle in cell life and death, whose damage endangers lipid protein synthesis, cell oxidative phosphorylation, ATP production in cell metabolism and regulation of cellular steady state. Mitochondrial morphology changes between fission and fusion (dynamical network), which is important in maintaining mitochondria function and quantity. Mitochondrial fusion is mediated by Mitofusin 2 and OPA1,[17,18] and its fission is regulated by dynamin-related protein (DRP1).[19] Mitochondrial fission and fusion balance is essential for maintaining the stability of its structure and function. When mitochondrial balance disorders, especially in divided state, mitochondrial membrane

*These authors contributed equally to this work.

Address correspondence to XiaoHong Tang, Division of Nephrology, West China Hospital of Sichuan University, Chengdu, Sichuan, China. E-mail: xhtng@126.com

potentially decreases, membrane permeability increases, ROS is produced, ATP generation goes down and a large number of apoptotic factors are released.[20,21] These ROS can further promote mitochondrial fission[22] and abnormal signal transduction pathways.[23–25] A large number of studies have shown that mitochondrial dysfunction plays an important role in the progression of renal disease including renal tubular injury, chronic kidney disease, and acute renal failure, which can lead to renal disease aggravation and progression.[26–28] More and more evidences suggested that the damage of mitochondrial morphological and function plays an important role in the development of DN.[29,30] Malik et al.[31] found that mitochondrial DNA copy number of peripheral blood in patients with DN is significantly higher than the DM group without nephropathy, which prompted that mitochondrion plays a key role in DN, namely, mitochondrial fission is increasing under HG. Hence, mitochondrial division may be one of the key points in DN progression.

DRP1, a member of the dynamin family of large GTPases, mostly locates in the cytoplasm, but is stimulated after fission stimuli to migrate to the mitochondria. Once there, DRP1 forms ring-like structures, which wrap around the scission site to constrict the mitochondrial membrane resulting in mitochondrial fission.[32,33] The level of DRP1 mRNAs is high in brain, moderate in skeletal and heart muscles, and low in pancreatic tissues.[34] Previous studies indicated that DRP1 is closely correlated with neurodegenerative diseases.[34,35] DRP1 was overexpressed in L5 dorsal root ganglion neurons under oxidative injury and apoptosis and then translocated to mitochondrial membrane, promoting mitochondrial fission, cytochrome C release, caspase system activation, and finally resulting in mitochondrial break-up and apoptosis.[36] Research on DRP1 is rare in kidney disease, and the mechanism is not clear; Broos et al.[20] found that DRP1 can translocate mitochondrial break-up and promote apoptosis when tubule cells get damaged. However, DRP1, mitochondrial fission protein, regulating mitochondrial division of functions has not been studied in progression of DN. Previous studies revealed that ECM accumulation in DN is closely associated with the activation of the p38 MAPK-signaling pathway, oxidative stress, and mitochondrial dysfunction.[16,37–41] Therefore, we predict that the abnormality of DRP1 affects mitochondrial function, and further influences p38 MAPK-signaling pathways, thus affecting DN.

This innovative research studied the location distribution of DRP1 in DN and that DRP1 participates in the regulation of mitochondrial function, thus resulting in increased oxidative radicals (ROS) release, activating P38-MAPK-signaling pathways, and affecting the increase of the ECM inversion. Thus, mitochondrial division inhibitor 1 (Midivi-1), a new DRP1 inhibitor, can alleviate renal cell apoptosis and acute kidney injury.[20] So, by taking the Midivi-1, the DRP-1-specific inhibitors, intervening in the translocation of DRP1 in HG, we future explore the relationship between DRP-1 and DN.

Methods

Cell culture

The rat GMC strain was provided by the China Center for Type Culture Collection (Wuhan University, Wuhan, Hubei, China, No. HBZT-1). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), streptomycin (100 μg/mL), penicillin (100 IU/mL), and 2 mM glutamine at 37°C in 95% air/5% CO_2. Rat GMCs were used for experiments after four to six passages. After culture, the medium was changed to DMEM with 5 mM glucose, 15 mM glucose, 30 mM glucose, or 25 mM mannitol, which was used as a hyperosmotic control. If necessary, the medium was changed every 48 h to maintain the appropriate concentration of glucose. We also injected Midivi-1 (50 μM in dimethyl sulfoxide;[42] Sigma, St. Louis, MO), an inhibitor of mitochondrial division, which is identified by Cassidy-Stone and colleagues and *N*-acetyl-L-cysteine (NAC), 3 Mm, which is commonly used to inhibit ROS to the medium of HG.

Electron microscopy of mitochondria

Primacy isolated cells and monolayer cultures were washed twice with PBS and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h. After rinsing twice with 0.2 M cacodylate buffer, monolayer cultures were cut in small sheets, whereas primary isolated cells were pelleted. Both cells in culture and primary isolated cells were then postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, treated with 2% uranyl acetate for 1 h, dehydrated in ethanol, and then embedded in Spurr's resin.[43] Ultra-thin sections were cut with an ultramicrotome (Ultracut, Reichert, Austria), and mitochondrial morphological changes were observed by transmission electron microscopy. Digital images were obtained using a Hitachi TEM transmission electron microscope (Hitachi, Japan). We used electron microscopy to observe the state of mitochondria, including fission in different medium (NG, HG, and HG+ Midivi-1).

Mitochondria isolation

Mitochondria were isolated from GMCs followed by multiple centrifugations at 700 *g*, 7800*g*, and 10,000 *g* in ice-cold isolation buffer containing 50 mM Tris/HCl, pH 7.4, 250 mM sucrose, 5 mM EDTA, and protease inhibitors (Sigma).[44] The mitochondrial protein concentration was determined by Bradford protein assay.[45] It is imperative that mitochondria used for proteomics studies are isolated by methodologies that result in functional and pure mitochondrial preparations. After isolation and determination of the mitochondrial protein concentration, the appropriate volume to achieve 1.0 mg protein is pipetted into a 1.5-mL microcentrifuge tube and mitochondria are spun at 12,500 rpm for 10 min to form a 1.0-mg protein pellet. The supernatant is removed and pellets are stored, and dried at −80°C before used in experiments. Mitochondria samples can then be resuspended in the appropriate buffer for proteomic experiments. Mitochondria are typically stored in suspension at a concentration of at least 10 mg proteins/mL.

ROS production measurement

At the end of the treatment schedule, cells were incubated with 10 μM 20,70-dichloro-dihydrofluorescein diacetate dye (DCF-DA; ABCAM) in culture media for 30 min at room temperature. Then, cells were washed with PBS,

trypsinized, resuspended in PBS supplemented with FBS, and analyzed for intracellular ROS production by flow cytometry (Becton, Dickinson and Company, Franklin Lakes, NJ).

Mitochondrial lipid peroxidation assessment

Mitochondrial lipid peroxidation was assessed by measuring the malondialdehyde (MDA) content by the thiobarbituric acid method.[46] The amount of MDA formed was calculated from the standard curve prepared using 1,1′,3,3′-tetramethoxypropane, and values were expressed as nmol/mg protein. The total mitochondrial protein concentration was determined using a bicinchoninic acid protein assay kit (Sigma, Santa Clara, CA). The activity and absorption were measured using a PerkinElmer Lambda Bio U/V spectrophotometer (Waltham, MA).

Western blot analysis for DRP1, p-P38MAPK, P38MAPK, and collagen IV

Protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA). Equal amounts of proteins from each sample were separated using 10% sodium dodecyl sulfate polyacrylamide gels electrophoresis and then transferred onto polyvinylidene fluoride membranes (Nu-PAGE; Invitrogen). The membranes were probed overnight using the corresponding antibodies: anti-phospho-p38 MAPK (1:2000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), anti-p38 MAPK (1:2000 dilution, Santa Cruz Biotechnology), and anti-collagen IV (1:1000 dilution, Sigma). The membranes were then incubated with a secondary antibody (1:10,000; Santa Cruz Biotechnology) for 2 h. Bands were visualized by enhanced chemiluminescence (Santa Cruz Biotechnology), and samples' loadings were normalized by immunoblotting with an anti-β-actin monoclonal antibody (1:2,000; Chemicon, Billerica, MA).

Statistical analysis

All values are expressed as means ± standard error of the mean. Multiple group means were compared by one-way analysis of variance. A p value of <0.05 was considered statistically significant. Statistical Product and Service Solutions 17.0 (SPSS Inc., Chicago, IL) statistical software was used for analysis.

Results

DRP1, ROS, and MDA expression in GMC

To test how DRP1, ROS, and MDA changes in different culture medium, Western blot analysis and flow cytometry were performed on the GMC prepared. Four groups of medium, respectively, are normal-glucose (NG, group A), mannitol (group B), 15 mM HG (15 mM HG, group C), and 30 mM HG (30 mM HG, group D). In group D (30 Mm HG), DRP1 expression (the total and that in mitochondria), ROS, and MDA were higher than those in group B (mannitol), group C (15 mM HG), and group A (NG). Western blot analysis illustrated that the expression of total DRP1 and that in mitochondria in groups C and D increased, and DRP1 in mitochondria in group D was statistically higher than that in group A ($p<0.05$) (Figure 1-III), which explained that DRP1 experienced translocation under the stimulation of HG, shifting from the cytoplasm to the mitochondrial membrane, because the role of DRP1 is to promote the mitochondrial fission, so we say HG influenced the fission of mitochondria by DRP1 translocation and further affected mitochondrial function.

ROS production was measured to observe the influence of DRP1 on mitochondrial function, which can reflect the degree of ROS in DN. As shown in Figure 1-IV, the level of oxidation products rose in groups C and D, and group D was statistically higher than group A ($p<0.05$). On the other hand, DRP1 translocation stimulated oxidative stress.

Mitochondrial lipid peroxidation was assessed by measuring the MDA, which can reflect its function. The level of MDA increased in groups C and D, and group D was statistically higher than group A ($p<0.05$) (Figure 1-V).

From Figure 1, we can see that the changes in DRP1, ROS, and MDA were consistent.

Pathological analysis of mitochondrial and DRP1, ROS, and MDA expression with Mdivi-1

Three groups of medium, respectively, are control group with NG (group A), 30 mM HG (30 mM HG, group B), and 30 mM HG with Midivi (group C). Midivi-1, the inhibition of DRP1, can suppress mitochondrial translocation from cytoplasm to mitochondrial membrane, and furthermore reduce ROS and MDA expression. DRP1 expression in group C decreased significantly compared to group B. As shown in Figure 2-II, the level of DRP1in group B was statistically higher than group A ($p<0.05$), but that in group C was less than group B ($p<0.05$). These can explain that Midivi-1 can reduce mitochondrial fission and protect its function by inhibiting DRP1 translocation.

Electron microscopy showed the most remarkable mitochondrial morphology changes in group B, with significant mitochondrial fission (Figure 2-III). The changes were more dramatic in group B than in groups A and C (Figure 2-III). A difference among the ROS and MDA contents in the three groups was observed: the highest ROS content in group B was 3.52 ± 0.35 times the A group and 1.25 ± 0.28 times the C group. The ROS content was significantly lower in group C than in groups B (Figure 2-III). Meanwhile, as aforementioned, mitochondrial MDA represents mitochondrial lipid peroxidation. The MDA content in group B was the highest among the three groups. The MDA content was lower in group C than in group B (Figure 2-III).

From Figure 2, we can see that the inversion of Midivi-1 can protect mitochondrial function by suppressing DRP1 translocation directly and ROS and MDA indirectly.

Expression of phospho-p38 protein

Phospho-p38, the upstream activator of p38 MAPK, exhibited a similar trend to DRP1.The groups of Figure 4-I and -II are same to Figure 1 and the groups of Figure 4-III and -IV are A:HG, B:NAG + HG, C:Midivi-1 + HG.

P-P38/p38 has higher levels in group D (HG) than in group A (NG) ($p<0.05$) (Figure 3-II). NAC is commonly used to inhibit ROS. But from Figure 3-III and -IV, we can see that p-P38/p38 is lower in groups B (NAG + HG) and C (Midivi-1 + HG) than group A (HG).

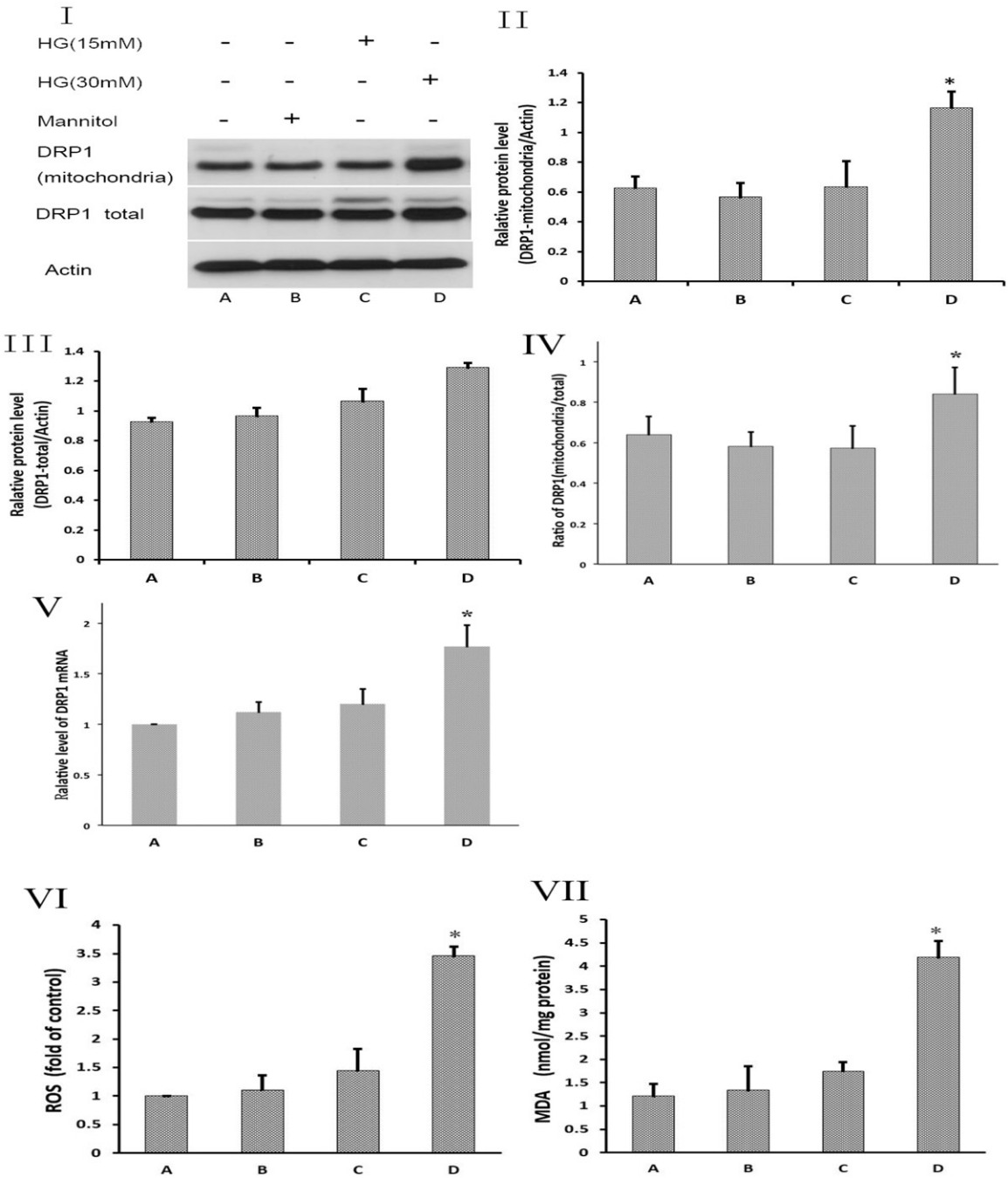

Figure 1. Hyperglycemia can stimulate DRP1 transposition and affect mitochondrial function. (I) Western blot of total DRP1 and DRP1 in GMC mitochondria. (II) Relative quantity of DRP1 in mitochondria (*$p < 0.05$ when compared with group A). (III) Relative quantity of total DRP1 in GMC (*$p < 0.05$ when compared with group A). (IV) Relative quantity of DRP1 mRNA in mitochondria (*$p < 0.05$ when compared with group A). (V) Relative quantity of total DRP1 mRNA in GMC (*$p < 0.05$ when compared with group A). (VI) Relative quantity of ROS (*$p < 0.05$ when compared with group A). (VII) Relative quantity of MDA in mitochondria (*$p < 0.05$ when compared with group).

Expression of collagen IV and phospho-p38 protein

Collagen IV is a main component of the ECM, and the expression of this protein can reflect the degree of renal damage. The group is same as in Figure 2. The groups in Figure 4 are same as in Figure 2. Western blot analysis shows that collagen IV expression significantly increased in group B. Collagen IV expression in group B (HG) was statistically higher than that in group A (the normal control group) and group C, which has Midivi-1 ($p < 0.05$) (Figure 4-II).

Figure 4 showed that Midivi-1 can obviously reduce the expression of collagen IV caused by hyperglycemia.

Figures 3 and 4 showed that phospho-p38 protein and collagen IV expression are increased under HG.

Discussion

DRP1, also called DNM1L protein, is the mitochondrial division essential protein whose excessive expression can promote mitochondrial fission and cytochrome C release and can further accelerate cell apoptosis. DRP1 is the critical factor in the regulation of mitochondrial shape, which has been approved in many diseases including Huntington's disease, Parkinsonism, pulmonary arterial hypertension, and

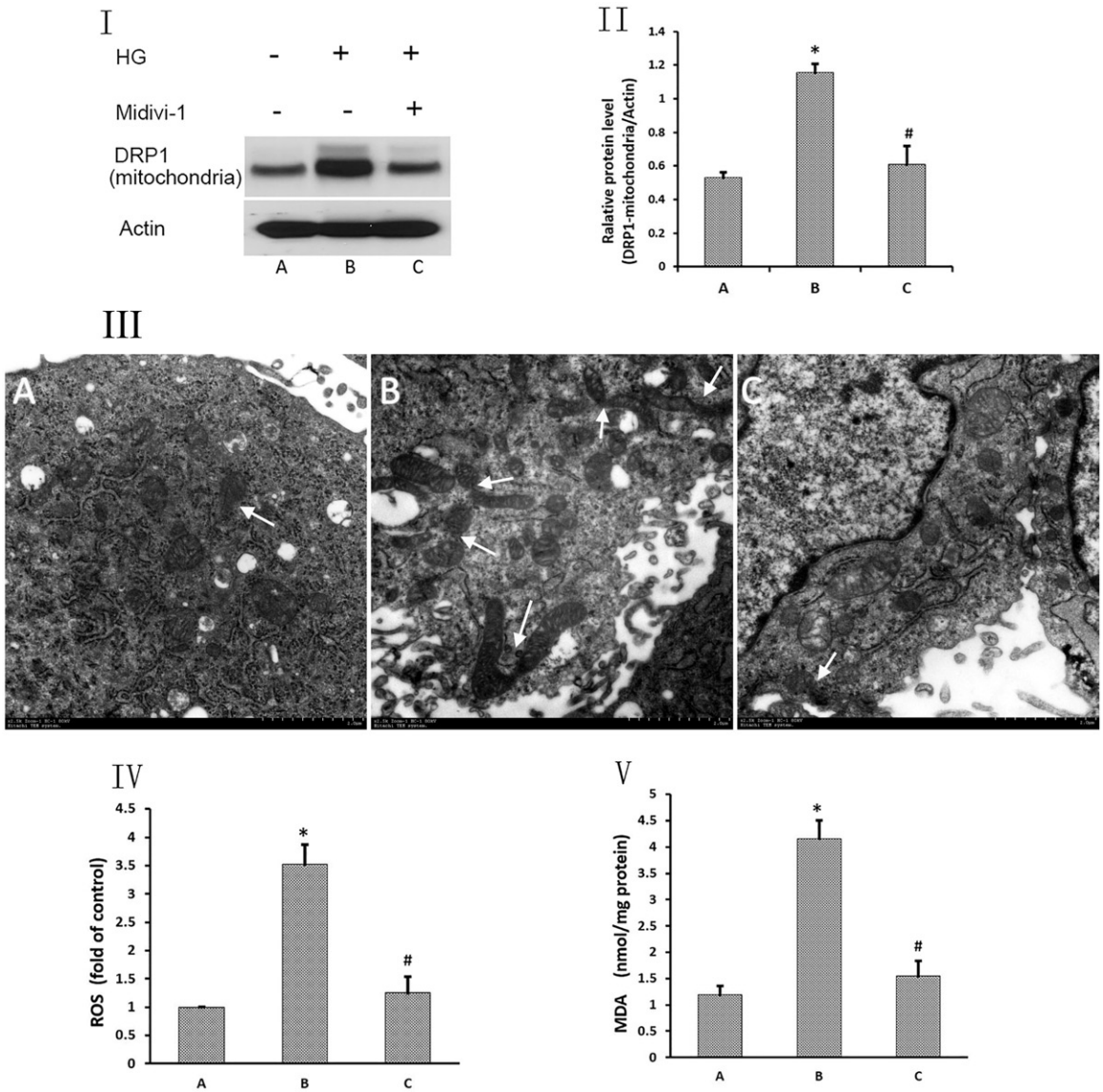

Figure 2. Midivi-1 can inhibit DRP1 transposition, reduce mitochondrial division, and improve mitochondrial function. (I) Midivi-1 inhibits the HG-produced transposition of DRP1 by Western blot. (II) Relative quantity of DRP1 in mitochondria (*$p < 0.05$ when compared with group A; #$p < 0.05$ when compared with group B). (III) Electron microscopy of mitochondrial division. Arrows: mitochondrial division. (B and C) Midivi-1 significantly suppressed the HG-induced division of mitochondria. (IV) Relative quantity of ROS expression levels (*$p < 0.05$ when compared with group A; #$p < 0.05$ when compared with group B). (V) Relative quantity of MDA production by mitochondria (*$p < 0.05$ when compared with group A; #$p < 0.05$ when compared with group B).

myocardial insulin resistance, and these studies mentioned that the expression of DRP1 is consistent with the increase in mitochondrial fission.[35,47–49] However, there is little research about the role of DRP1 in renal disease including DN. Peng[50] found that hyperglycemia increased the expression of DRP1 and yielded DRP1-induced mitochondrial fission to cause mitochondrial fragmentation and apoptosis in INS-1-derived cells. Mounting evidences indicate that DRP1 can stimulate mitochondrial fission and perturb mitochondrial membrane permeability, and further cause apoptosis of pancreatic β cells.[49,50] However, there is no research on DRP1 in DN, so we mention that DRP1 plays a critical role in early stage of DN. A variety of pathogenic factors under diabetes status stimulate DRP1 transmigrate to mitochondrial outer membrane, release large ROS, and result in apoptosis of mesangial cell, forming early DN. Therefore, this study is pioneering about DRP1 in DN.

Our data demonstrate that mitochondrial DRP1 expression is significantly increased in HG (30 Mm) medium (Figure 1), while the total DRP1 increased but has no statistically significance, which explained that HG plays its role through promoting DRP1 translocation rather than increasing DRP1 expression. Oxidative stress is the key to promote DN progression, and DRP1 is the critical factor of ROS. DRP1 maintains the morphology of mitochondria by influencing the distribution of microtubules, mainly located in cytoplasm, and is the essential protein in mitochondrial division.

Similarly, Wang et al.[51] reported that the increase in DRP1 translocation to mitochondrial outer membrane can promote its fission in podocyte and endothelial cell. Peng et al.[50] studied that the expression of DRP1 can explain apoptosis caused by mitochondrial fission, and the suppression of DRP1 can protect beta cell. Labrousse's study showed that DRP1 in the cytoplasm metastasize to position of mitochondrial outer

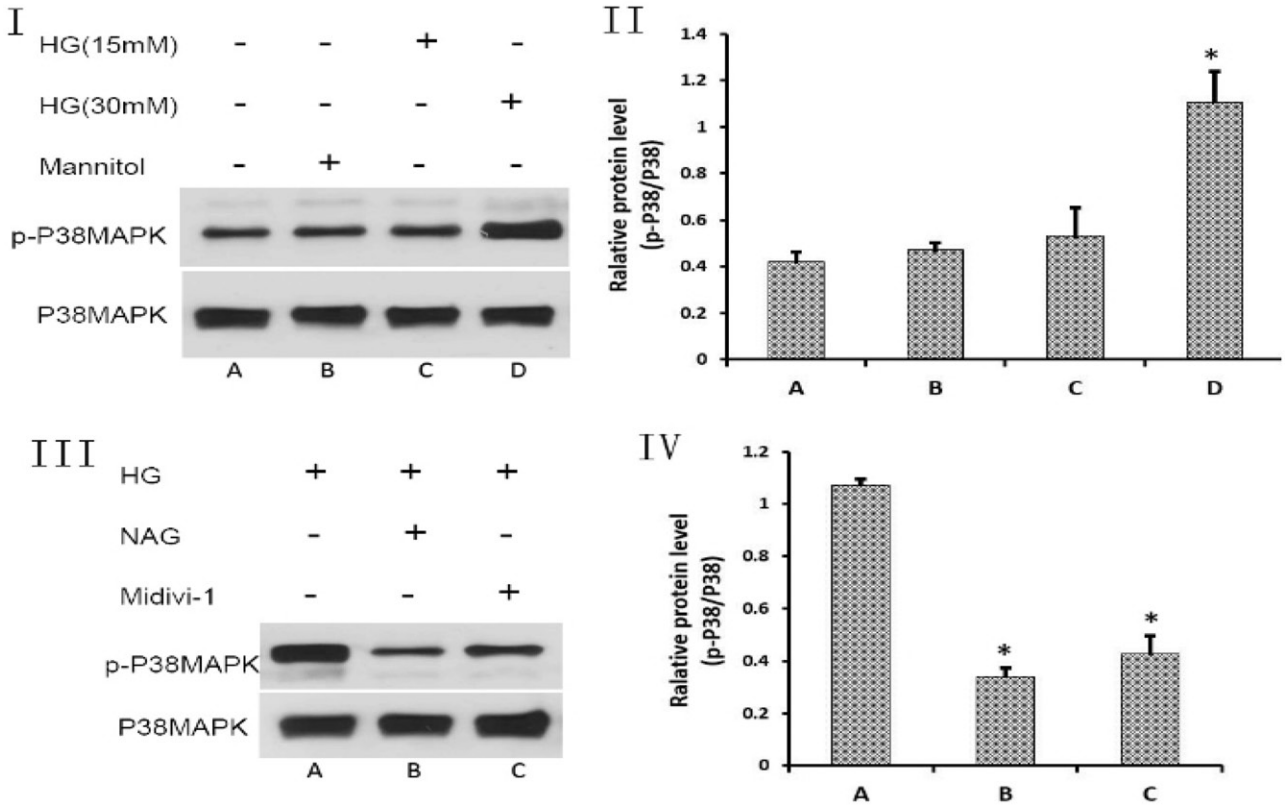

Figure 3. HG can stimulate p38 phosphorylation. Midivi-1 can reduce the production of ROS and suppress p38 phosphorylation by inhibiting DRP1 transposition. (I) Protein expression of phospho-p38 and p38 in GMC (Western blot). (II) Ratio of p-p38/p38 (*$p < 0.05$ when compared with group A). (III) Ratio of p-p38/p38 with intervention by NAG and Midivi-1 (group A: HG; group B: intervention with NAG; group C: intervention with Midivi-1). (IV) Relative quantity of phosphor-38 expression levels (*$p < 0.05$ when compared with group A).

membrane (MOM), which enrich the divided sites-Fis1. And Fis1 enables multiple DRP1 forming ring structure around mitochondria, hydrolyzes GTP to change the distance or Angle, and compressed mitochondrial until its breakage, then reduces independent fragmented mitochondria, thus plays its role. Mesangial cell depicts the phenomenon combining our research, therefore, DRP1 in cytoplasm transferred to mitochondrial outer membrane and resulted in its fission under the condition of high sugar. Figure 1 also shows that the changes in the trend of DRP1 in mitochondria, ROS, and MDA are same, namely, under the condition of high sugar, ROS and MDA increased significantly. Some research suggests that the expression of DRP1 before can decrease the mitochondrial membrane potential, promote cytochrome C release, activate proteinase 3 and the production of ROS, and further facilitate the oxidative stress and mitochondrial necrosis.

Electron microscopy images displayed that mitochondrial morphology is different under normal conditions and HG conditions, and the mitochondrial fission is most obvious in HG. Midivi-1, a new inhibitor for DRP1, can inhibit the DRP1 translocation, thereby inhibiting mitochondrial fission. To further define the role of DRP1, we combined the Midivi-1 in culture (group C in Figure 2), and in this group, DRP1 on mitochondria was significantly reduced, and ROS and MDA expression were also significantly inhibited. Guo[52] studied that DRP1, as mitochondrial fission, targeted the mitochondrial outer membrane under the condition of oxidative stress, thereby causing mitochondrial fission, followed by mitochondria and cell apoptosis, and the inhibition of DRP1 function prevented its occurrence.[52] We applied the Midivi-1 in our experiments, which can arrest DRP1 translocation. So, DRP1 translocation is the key in mitochondrial fission, which was caused by HG in GMC, and this can lay the foundation for further clinical studies in the future; in other words, inhibiting the DRP1 translocation may block the development of DN.

Meanwhile more and more evidences indicate that ROS plays a key role in the progression of DN and increased ROS can activate pro-fibrotic factors including TGF-β through P38MAPK, which can promote the synthesis of collagen IV (main ingredient of ECM) and fibrin connection protein, thus causing an increase in ECM, and forming early DN.[16,37–40] Our experiments also illustrated this trend, thus we assumed that DRP1 translocation leads to the activation of the p38. We observed that the group with Midivi-1 can suppress this signal path, and further inhibit mitochondrial division (by ROS regulation). So, we interpreted that DRP1 translocation is the main reason for activation of p38-signaling pathway and increase in collagen IV.

As far as we know, this study is the first to determine that HG can promote mitochondrial fission by regulating DRP1 transposition, and the result is optimistic. Midivi-1, as DRP1 translocation inhibitors, can reduce mitochondrial fission and protect mitochondrial function. DRP1 affects p38 MAPK-signaling pathway by regulating mitochondrial function and adjusts the expression of ROS; and this process can be

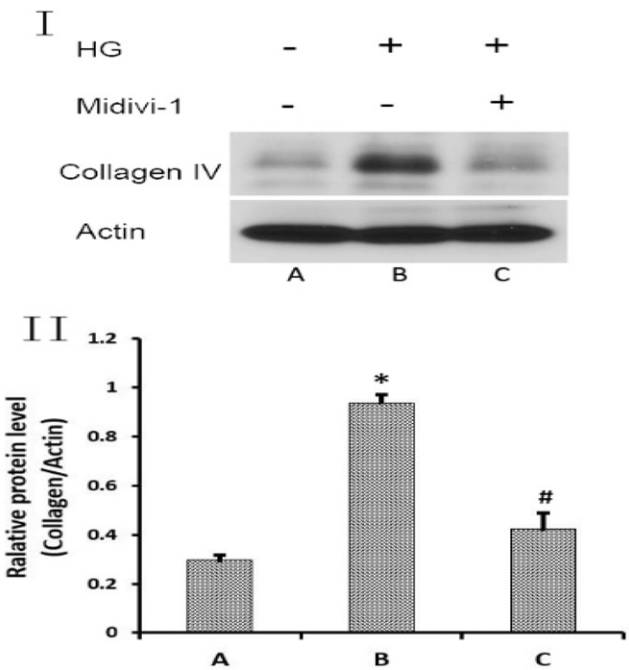

Figure 4. Intervention of Midivi 1 can reduce the expression of collagen 4. (I) Protein expression of collagen IV (Western blot). (II) Relative protein level of collagen IV ($*p < 0.05$ when compared with group A; $\#p < 0.05$ when compared with group B).

suppressed by Midivi-1; therefore, it indicates that Midivi-1 can block this pathway, reduce ROS expression, and further inhibit mitochondrial fission. The relationship among the DRP1, ROS, and p38-signaling pathway is the key of DN. The study also confirmed that the activation of p38 can lead to the synthesis of collagen IV. Inhibition of DRP1 translocation can be used as therapy targets in early diabetes intervention.

Currently, there are some limitations of this study: (1) concrete mechanisms of DRP1 translocation is not yet clear; (2) animal testing has not been carried out. Further research will focus on these unresolved issues, and continue to explore the role of DRP1 and translocation suppression in the early intervention in the DN.

In short, DRP1 translocation can cause pathological change of glomerulus mesangial cell in HG. DRP1 is one of the destructive elements in DN, which can promote mitochondrial fission, cause oxidative stress, and accelerate the activation of p38-signaling pathway. Midivi-1 can inhibit above changes by suppressing DRP1 translocation, and in future, intensive study of the mechanism of DRP1 and Midivi-1 is an effective intervention direction for the early DN.

Declaration of interest

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