Original Paper

Attenuation of Glomerular Endothelial Cells from High Glucose-Induced Injury by Blockade of MAD2B

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Key Words

MAD2B • High glucose • Glomerular endothelial cells

Abstract

Background/Aims: To assess the role of mitotic arrest-deficient 2-like protein 2 (MAD2B) in high glucose-induced injury in mouse glomerular endothelial cells (GEnCs). **Methods:** GEnCs were cultured *in vitro*, and MAD2B protein levels were measured by Western blot in cells stimulated with high glucose (30 mM) for various periods of time. *MAD2B* and scrambled shRNA were introduced into GEnCs by liposomal transfection. Cell proliferation, apoptosis, nitric oxide (NO) production, and monolayer permeability were then measured in cells grown in the following conditions: control, high glucose treatment, *MAD2B* shRNA transfection with high glucose treatment, and scrambled shRNA transfection with high glucose treatment. **Results:** High glucose increased the protein levels of MAD2B in GEnCs. Compared with control cells, apoptosis was increased by high glucose treatment, which was attenuated by transfection with *MAD2B* shRNA transfection. Cells treated with high glucose produced less NO than control cells, whereas *MAD2B* shRNA transfection increased NO production. Cell monolayer permeability was enhanced in high glucose treated cells, but *MAD2B* shRNA transfection reduced permeability. **Conclusion:** High glucose levels induced the expression of MAD2B in GEnCs, whereas suppressing its expression reduced high glucose-induced endothelial cell apoptosis and high permeability, and promoted cell proliferation and NO production.

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Introduction

Diabetic nephropathy (DN) is one of the most common microvascular complications of diabetes mellitus, it has also become the second leading cause of end stage renal disease (ESRD) in China [1]. Due to the confounding metabolic syndrome, it is more difficult to treat diabetes-induced ESRD than other kidney diseases. Therefore, early prevention is important for delaying the development of DN.

The pathogenesis of DN is not fully understood. A large body of research suggests that hyperglycemia causes abnormalities in hemodynamics and glucose metabolism, which lead to kidney damage and pathogenic changes in the resident cells of the kidney glomeruli. The role of glomerular endothelial cells (GEnCs) in the development of DN has become the focus of many recent studies [2]. Because of their direct contact with the circulation, vascular endothelial cells directly receive and respond to metabolic, biochemical, and hemodynamic signals in the blood. A number of phenotypic changes have been reported in GEnCs under hyperglycemic conditions, including suppressed cell proliferation and increased apoptosis [3], altered endothelial nitric oxide synthase (eNOS) activity and nitric oxide (NO) production [4], enhanced cell monolayer permeability due to cytoskeletal changes [5], the secretion of cytokines such as angiotensin II (Ang II), transforming growth factor β (TGF β), and interleukin 6 (IL-6) [6-8], and disruption of the cell surface glycocalyx [9]. Because endothelial cells are sensitive to blood glucose levels, hyperglycemia-induced endothelial injury and dysfunction form the initial step toward diabetic vascular complications. However, the exact molecular mechanisms underlying high glucose-induced GEnC dysfunction are not completely elucidated.

Previous data from our laboratory suggest that mitotic arrest-deficient 2-like protein 2 (MAD2B, also termed MAD2L2) is involved in the development of DN. MAD2B acquired its name due to its 23% sequence identity and 54% similarity to mitotic arrest-deficient 2 (MAD2) at the amino acid level [10]. Both of these proteins contain a HORMA structural domain, which is involved in protein-protein interactions, protein oligomerization, and chromatin state recognition. A yeast two-hybrid screen revealed that MAD2B interacts with CDH1 and CDC20, both of which are essential activators of the anaphase-promoting complex/cyclosome (APC/C). MAD2B regulates cell cycle progression by binding to CDH1 or CDC20 and inhibiting the activity of APC/C [11, 12]. MAD2B levels are upregulated in many malignant tumors, and correlate with tumor stage and poor prognosis, probably due to mitotic failure and genetic instability caused by the inhibition of APC/C [13-15]. We previously used immunohistochemistry to demonstrate that MAD2B protein levels were increased in the kidney glomeruli of streptozotocin (STZ)-induced type 1 diabetic mice, the db/db model of type 2 diabetes, and kidney biopsies of patients with diabetic nephropathy. In podocytes cultured *in vitro*, high glucose induced the expression of MAD2B, which mediated high glucose-induced injury by affecting the expression of APC/C substrates. In addition, the overexpression of MAD2B accentuated high glucose-induced podocyte injury and apoptosis, whereas inhibiting the expression of MAD2B attenuated such injury and apoptosis (paper in process). Despite the important role of endothelial cells in DN, the role of MAD2B in endothelial cells has not been studied. In the current study, we investigated the role of MAD2B in high glucose-induced GEnC injury, and discussed its potential association with DN.

Materials and Methods

Cell culture and treatment

Murine glomerular endothelial cells (GEnCs) were purchased from Cell Biologics Company (C57-6014G) and were cultured according to the manufacturer's protocol and previously reports [16]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco Invitrogen, LifeTechnologies, Scotland, UK) supplemented with 10% fetal bovine serum (FBS, Gibco Invitrogen). For high glucose treatment, cells were

dissociated with 0.25% trypsin-EDTA solution (Gibco Invitrogen) for 1–2 min at 37°C. As soon as the cells detached, cell culture media supplemented with 10% FBS were added, and the cells were plated in six-well plates coated with a gelatin-based coating solution in a humidified, 5% CO₂ incubator at 37°C. Twelve hours later, cells were incubated in DMEM without FBS for another 24 h. Cells were then incubated in 5.5 mM D-glucose (control, normal glucose [NG] group), NG plus mannitol (5.5 mM glucose +24.5 mM mannitol; an osmotic control), or 30 mM D-glucose (high glucose [HG] group) for 12, 24, or 48 h. MAD2B levels were then determined by western blot as described below.

Western blot

Western blot was performed as described previously [17]. Briefly, protein concentrations were determined (BCA Protein Assay Kit, Beyotime, Shanghai), and 70 µg protein samples were separated on 12% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Sigma-Aldrich, St. Louis, MO, USA). Non-specific binding was blocked by incubating membranes in 5% fat-free milk in TBS buffer containing 0.1% Tween 20 (TBS-T) for 1 h. Membranes were then incubated overnight at 4°C with primary antibodies against MAD2B (1:1000, Rockland Immunochemicals Inc. Gilbertsville, PA, USA) and β-actin (1:4000, Santa Cruz Biotechnology, CA, USA). After washing, the blots were incubated for 1 h at room temperature (RT) with horseradish peroxidase (HRP)-labeled secondary antibodies (1:4000). Immunoreactive bands were developed using an enhanced chemiluminescence (ECL) Western blot detection reagent (Thermo Scientific, MA, USA). β-actin was used as the loading control. The density of each band was quantified using Image J software (NIH, Bethesda, MD, USA).

Transient transfection of recombinant plasmids into GEnCs using liposomes

GEnCs were plated into six-well plates 24 h before transfection, and were transfected when they became 80% confluent. Thirty minutes before transfection, the culture media were changed to OPTI-MEM (Gibco, USA). For transfection, 4 µg of plasmid DNA (MAD2B shRNA or scrambled shRNA) was added to 50 µl OPTI-MEM. In a separate tube, 8 µl of Lipofectamine 2000™ (lipo 2000, Invitrogen, CA, USA) was added to 50 µl of OPTI-MEM; both tubes were mixed and left to stand at RT for 5 min. The diluted DNA and lipo 2000 solutions were then combined, gently mixed, and left to stand at RT for 20 min. The mixture was then added into 6-well plates, which were gently shaken and then placed back into the incubator. Six hours after transfection, the media were replaced with complete media.

Cell proliferation assay

GEnCs were transfected in six-well plates as described above, and were dissociated into single-cell suspensions 6 h after transfection. The cells were re-plated into 96-well plates (100 µl/well, ~5,000 cells/well) and incubated for 12 h. The media were then replaced with 100 µl/well fresh medium, and the cells were divided into four treatment groups: control, high glucose alone, high glucose plus scrambled shRNA transfection, and high glucose plus MAD2B shRNA transfection. For GEnCs proliferation assay, a colorimetric BrdU Cell Proliferation ELISA Kit (Abcam, Cambridge, UK) was used as described previously [18]. After 48 h treatment of high glucose, BrdU was added 24 hours before the end of the incubation period. After that, cells were fixed and BrdU content was assessed using a monoclonal anti-BrdU antibody according to the manufacturer's instructions. The absorbance at 450 nm was then measured using a microplate reader (TECAN, Männedorf, Switzerland).

Apoptosis analysis using flow cytometry

GEnCs were transfected in six-well plates as described above, and were then divided into four treatment groups 6 h after transfection: control, high glucose alone, high glucose plus scrambled shRNA transfection, and high glucose plus MAD2B shRNA transfection. Cells were collected for analysis after 48 h of high glucose treatment. The culture media containing floating cells were removed from the wells and transferred into labeled tubes. The attached cells were then dissociated with 0.25% trypsin (no EDTA) and transferred into the same labeled tubes. Cells were centrifuged at 2,000 rpm for 5 min at RT, the supernatant was discarded and the cells were washed twice with PBS. The cells were centrifuged again, and resuspended in 500 µl of binding buffer. To stain apoptotic cells, 1 µl of annexin V-APC was added, followed by 5 µl of 7-AAD buffer (Nanjing KeyGEN Biotech, China). Cells were stained in the dark for 15 min, and then analyzed by flow cytometry (BD, Biosciences, CA, USA) within 1 hour.

Quantification of NO levels in culture media (nitrate reductase assay)

GEnCs were transfected in six-well plates as described above, and were divided into four treatment groups: control, high glucose alone, high glucose plus scrambled shRNA transfection, and high glucose plus *MAD2B* shRNA transfection. The culture media were collected after 24 or 48 h of high glucose treatment, and the levels of NO in the media were measured using a NO quantification kit (Nanjing Jiancheng Biotech, China) following the manufacturer's instructions.

Cell monolayer permeability assay

GEnCs were transfected in six-well plates as described above, dissociated into single cell suspensions of 2×10^6 cells/ml after 6 h, and then plated into the upper chamber of Transwell plates in which the membranes (polycarbonate, pore size 0.4 μ m, 12 mm diameter) were coated with the matrix from an *in vitro* vascular permeability assay kit (Millipore, Bedford, MA, USA). Cells were divided into four treatment groups: control, high glucose alone, high glucose plus scrambled shRNA transfection, and high glucose plus *MAD2B* shRNA transfection. A total of 500 μ l of the appropriate media were added to the lower chambers. When cells reached confluency 48 h later, the media were removed from both chambers without disturbing the cell monolayer; 150 μ l of FITC-dextran (1 mg/ml) was added to the upper chamber, and 500 μ l culture medium (without phenol red) was added to the lower chamber. The plates were left to stand at RT for 5 min, and then 100 μ l of the medium was removed from the lower chamber to measure the fluorescence intensity using a fluorescence spectrometer (Shanghai Jingke, China). The relative permeability of cells in each treatment group was calculated as the ratio of the mean intensity of the experimental group to the mean intensity of the control group.

Statistical analysis

All data are presented as means $\pm$ standard deviations ($M \pm SD$), and GraphPad Prism 5 was used for all statistical analyses. One-way analysis of variance (ANOVA) was used to compare differences among groups, and $p < 0.05$ designated to indicate statistical significance.

Results*High glucose upregulates MAD2B in cultured GEnCs*

Murine GEnCs were incubated in high glucose (30 mM) for 0, 12, 24, or 48 h, and *MAD2B* protein levels were measured by Western blot. *MAD2B* levels were significantly higher at all time points (12–48 h) following high glucose stimulation, and peaked at 24 h (Fig. 1A, B). However, *MAD2B* levels were unaffected by treatment with a high concentration of mannitol (24.5 mM mannitol + 5.5 mM glucose) (Fig. 1C, D), suggesting that the effect of high glucose on *MAD2B* expression was not simply due to high osmotic pressure.

MAD2B shRNA effectively blocks MAD2B expression

GEnCs were transfected with recombinant plasmids expressing *MAD2B* or scrambled shRNA, and Western blot was used to assess transfection efficiency after 48 h. Compared with untransfected control cells, *MAD2B* protein levels were significantly lower in those transfected with *MAD2B* shRNA, whereas expression was unchanged in those transfected with scrambled shRNA (Fig. 2).

MAD2B blockade promotes proliferation of high glucose-treated GEnCs

GEnCs were treated as described above, and incubated for 48 h; cell proliferation was then determined using a BrdU Cell Proliferation ELISA Kit. The proliferation rate of high glucose-treated cells was declined significantly at 48 h comparable to that of control cells (Fig. 3). *MAD2B* shRNA-transfected cells proliferated faster under a high glucose environment than scrambled shRNA transfected cells, but remained slower than control cells (Fig. 3).

MAD2B blockade reduces apoptosis of high glucose-treated GEnCs

GEnCs were treated as described above, and apoptosis was analyzed using flow cytometry after 48 h. Fig. 4A shows representative dot plots from each group. The lower

Fig. 1. High glucose increases MAD2B protein levels in glomerular endothelial cells (GEnCs). GEnCs were stimulated with high glucose (HG) (A–B) or high mannitol (C–D) for 0 (Ctrl), 12, 24, or 48 h, and MAD2B expression was analyzed by Western blot. A, C, Representative images of western blots are shown. B, D, Bar graphs showing the results of statistical analysis. * $p < 0.05$ vs. Ctrl ($n = 4$ in B, and $n = 3$ in D).

Fig. 2. MAD2B shRNA transfection decreases MAD2B protein levels in GEnCs. GEnCs were left untransfected (Ctrl), or were transfected with scrambled shRNA (Scra) or MAD2B shRNA (shRNA); MAD2B levels were then analyzed by Western blot. A, Representative Western blot images. B, Bar graph showing the results of statistical analysis. * $p < 0.05$ vs. Ctrl ($n = 3$).

Fig. 3. Knockdown of MAD2B induces GEnC proliferation. GEnCs were grown in normal glucose (Ctrl), high glucose (HG), and/or transfected with scrambled shRNA (scra) or MAD2B shRNA (shRNA). Cell proliferation was then assessed after 48 h of high glucose treatment. * $p < 0.05$ vs. Ctrl; # $p < 0.05$ vs. HG + scra at the same time point ($n = 4$).

left quadrant contains live cells, the upper left quadrant includes cells that were damaged mechanically, the lower right quadrant represents early apoptotic cells, and the upper right quadrant contains late apoptotic and necrotic cells. The apoptotic index was calculated as the sum of the percentages of cells in the lower and upper right quadrants. Compared with

Fig. 4. Knockdown of *MAD2B* reduces apoptosis in GEnCs. GEnCs were grown in normal glucose (Ctrl), high glucose (HG), and/or transfected with scrambled shRNA (scra) or *MAD2B* shRNA (shRNA), and apoptosis was analyzed using flow cytometry. A, Representative dot plots showing annexin V-APC on the x-axis and 7-AAD on the y-axis. B, Bar graph showing the results of statistical analysis. * $p < 0.05$ vs. Ctrl; # $p < 0.05$ vs. HG + scra ($n = 3$).

Fig. 5. Knockdown of *MAD2B* induces nitric oxide (NO) production in GEnCs. GEnCs were grown in normal glucose (Ctrl), high glucose (HG), and/or transfected with scrambled shRNA (scra) or *MAD2B* shRNA (shRNA). The levels of NO secreted into the culture media were then analyzed 24 or 48 h after high glucose treatment. * $p < 0.05$ vs. Ctrl; # $p < 0.05$ vs. HG + scra at the same time point ($n = 6$).

Fig. 6. Knockdown of *MAD2B* reduces the monolayer permeability of GEnCs. GEnCs were grown in normal glucose (Ctrl), high glucose (HG), and/or transfected with scrambled shRNA (scra) or *MAD2B* shRNA (shRNA), and cell monolayer permeability was analyzed 48 h after high glucose treatment. * $p < 0.05$ vs. Ctrl; # $p < 0.05$ vs. HG + scra ($n = 3$).

control cells, the apoptotic index was significantly higher in cells treated with high glucose, whereas *MAD2B* shRNA-transfected cells had a lower apoptotic index than scrambled shRNA-transfected cells under the same high glucose conditions (Fig. 4B). This suggests that high glucose induces apoptosis in GEnCs, but that silencing *MAD2B* inhibits this effect.

MAD2B blockade induces NO production in high glucose-treated GEnCs

A nitrate reductase assay was used to measure the amount of NO secreted into the culture medium by cells in treatment groups described above. Measurements were taken after 24 or 48 h of high glucose treatment. Compared with control cells, the NO producing ability declined in GEnCs after high glucose treatment at both time points, whereas cells transfected with *MAD2B* shRNA secreted more NO than scrambled shRNA transfected cells under the same high glucose conditions (Fig. 5).

MAD2B blockade reduces monolayer permeability of high glucose-treated GEnCs

FITC-dextran was used as a tracking reagent in confluent GEnCs to assess cell monolayer permeability (Fig. 6). High glucose treatment significantly increased GEnCs monolayer permeability, whereas *MAD2B* shRNA transfection partially reversed this effect. This suggests that inhibiting *MAD2B* expression using shRNA could alleviate the increased endothelial permeability induced by high glucose.

Discussion

MAD2B is a member of the mitotic arrest deficient (*MAD*) family [10]. It interacts with many cellular proteins via its HORMA structural domain to regulate multiple cellular functions. Recently, a yeast two-hybrid screen revealed that *MAD2B* interacts with CDH1 and CDC20 [11], which both activate the APC/C complex. APC/C is an E3 ubiquitin ligase that plays a key role in the degradation of its target proteins via the ubiquitin-proteasome pathway. It regulates cell cycle progression by targeting cell cycle proteins, such as cyclin B1, cyclin A, p21, and p27, for polyubiquitination and subsequent proteasomal degradation [19]. *MAD2B* can inhibit APC/C activity by binding to CDH1 and CDC20; therefore, it is also involved in cell cycle control. In addition, *MAD2B* interacts with TCF4 and regulates the Wnt/ β -catenin signaling pathway [20]. Most studies on *MAD2B* have focused on its role in cancer and neurological diseases [13-15, 21-23], but it is still unknown about its expression and function in the kidney.

Because of their unique structure and function, the role of glomerular endothelial cells in kidney diseases has attracted significant attention. In many ways, DN may be viewed as a disease of the endothelial cell [24]. During DN, hyperglycemia and products of abnormal glucose metabolism activate a number of signaling molecules, transcription factors, and inflammatory mediators that together form a complex signaling network that induces glomerular endothelial dysfunction and injury [25]. The major changes in endothelial cell structure and function under high glucose that lead to progressive fibrosis, reduced capillary perfusion, and diminished GFR [26]. In the current study, we demonstrated that *MAD2B* levels were upregulated in GEnCs by high glucose, peaking at 24 h, and that this effect was not caused by high osmotic pressure.

Over the last few years, many *in vitro* studies focusing mainly on human or animal endothelial cells of kidney, retina, myocardium, and human umbilical vein endothelial cells (HUVECs) showed that hyperglycemia can induce endothelial cell apoptosis [27-29]. These mechanisms of apoptosis include oxidative stress, increased intracellular Ca^{2+} , mitochondrial dysfunction, changes in intracellular fatty acid metabolism, activation of mitogen activated protein kinases (MAPK) signaling pathways, and impaired phosphorylation activation of the protein kinase Akt [30-32]. In our study, we found hyperglycemia induced GEnCs apoptosis, in addition, we also observed that *MAD2B* shRNA reduced high glucose-induced apoptosis. Previous studies revealed that high glucose could induce the expression of *MAD2B* in podocytes and neurons, and that *MAD2B* mediates high glucose-induced apoptosis [22, 23]. Mechanistically, the binding of *MAD2B* to CDH1 inhibits APC/C activity, leading to the abnormal accumulation of the APC/C substrate cyclin B1. The cyclin B1-CDK1 complex can promote the phosphorylation of the apoptotic protein BAD at serine 128, which in

turn induces apoptosis [22, 23]. It is possible that MAD2B mediates high glucose-induced apoptosis in GEnCs via a similar mechanism.

In vitro, high glucose has been demonstrated to delay endothelial cell replication [33]. Culturing both human aortic endothelial cells (HAECs) and human umbilical vein endothelial cells (HUVECs) cells in high glucose increases the production of superoxide anion and also inhibits cell proliferation [34]. The results of our cell proliferation assay revealed that treatment with high glucose for 48 h inhibited GEnC proliferation, but that depleting MAD2B with shRNA promoted proliferation. In other tissues, studies assessing on the role of MAD2B in cell proliferation yielded conflicting results. In ovarian clear cell carcinoma cells, depleting MAD2B using shRNA reduced cell proliferation [21]. However MAD2B gene knockdown had no effect on the growth of nasopharyngeal carcinoma cells [15]. In intestinal epithelial cells infected with *Shigella*, MAD2B binds to IpaB, an effector protein secreted by *Shigella*, which releases MAD2B from CDH1 and CDC20 and leads to the over-activation of APC/C by CDH1 and CDC20. Therefore, *Shigella* disrupts the timely activation of APC/C and normal mitosis, causing intestinal epithelial cells to lose their ability for rapid self-renewal, which is their innate defense mechanism for controlling bacterial infection [35]. These results suggest that the role of MAD2B in cell proliferation is cell type-specific, probably because MAD2B binds to and regulate proteins from different signaling pathways in different cells.

In addition to partially reversing the effect of high glucose on cell proliferation and apoptosis, depleting MAD2B in GEnCs using shRNA also reversed the effect of high glucose on NO synthesis and monolayer permeability via unknown mechanisms. NO is a short-lived gaseous lipophilic molecule and is a paracrine mediator formed from its precursor L-arginine, which includes three NOS isoforms: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) [36]. NO is an important endothelium-derived mediator and with multiple actions, such as, vasodilation and antiproliferative, permeability-decreasing, and anti-inflammatory properties [37, 38]. Previous studies have provided conflicting results for the NO expression in diabetes. Most reports suggested that NO production is increased in early diabetes but decreased in advanced nephropathy [36]. Both eNOS and nNOS can contribute to the altered NO production, particularly the first [36]. Our study showed that HG decreased NO production, which is consistent with previous reports by Cheng and Hoshiyama et al [4, 38]. Decreased NO production and bioavailability might be associated with overproduction of superoxide and L-arginine deficiency, increased in microvessel permeability, resulting in impaired endothelial function [4, 36], and which, may contribute to DN progression [37, 39]. MAD2B can interact with many proteins through its HORMA domain, executing a diverse array of biological functions. Some of the signaling pathways regulated by MAD2B might affect eNOS activity and cytoskeletal structure, which in turn regulate NO production and endothelial permeability, respectively.

In summary, this study demonstrated that high glucose induced MAD2B expression in GEnCs, and that depleting MAD2B using shRNA could alleviate high glucose-induced endothelial injury, including effect on cell proliferation, apoptosis, NO production, and monolayer permeability. These data suggest that MAD2B mediates high glucose-induced GEnC injury. This is the first report describing a role for MAD2B in kidney glomerular endothelial cells; however its mechanism of action awaits further investigation.

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Disclosure Statement

All of the authors declared that there was no conflict of interest.

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