

Betaine alleviates high glucose-induced mesangial cell proliferation by inhibiting cell proliferation and extracellular matrix deposition via the AKT/ERK1/2/p38 MAPK pathway

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Abstract. Diabetic nephropathy (DN) is a major cause of chronic renal failure in diabetic patients worldwide. Betaine, a zwitterionic quaternary ammonium salt compound, is involved in numerous biological processes. The present study aimed to investigate the effects of betaine on mouse mesangial cells (MMCs) cultured under high glucose (HG) conditions and its underlying mechanisms. MMCs were treated with betaine under HG conditions. Cell proliferation and the cell cycle distribution were investigated with an MTT assay and flow cytometry, respectively. Western blotting and reverse transcription-quantitative polymerase chain reaction analyses were applied to respectively determine protein and mRNA expression levels. The results suggested that betaine decreased cell proliferation in a dose-dependent manner, while G1-phase arrest was significantly induced in MMCs. Compared with the control group, the expression levels of p21 and p27 decreased under HG conditions, but were reversed by betaine. Furthermore, the expression levels of fibronectin and type IV collagen were significantly decreased in cells treated with betaine compared with the HG group. Additionally, betaine decreased the phosphorylation of Akt, extracellular-signal-regulated kinase (Erk)1/2 and p38 mitogen-activated protein kinase (MAPK), but was enhanced under HG conditions. Overall, the results of the present study indicated that betaine serves a protective role in HG-induced MMCs by inhibiting cell proliferation and

extracellular matrix deposition via regulating regulation of the Akt/Erk1/2/p38 MAPK signaling pathway.

Introduction

Diabetic nephropathy (DN), as a diabetic microvascular complication, is mainly responsible for chronic renal failure in diabetic patients worldwide (1,2). Mesangial cell abnormalities and the deposition of extracellular matrix (ECM) proteins, such as fibronectin and collagen, are the main pathological hallmarks of DN (3). It has been reported that the proliferation of mesangial cells serves a vital role in the initiation and development of DN (4). Under high glucose (HG) conditions, glomerular mesangial cell dysfunction, followed by imbalances in ECM protein secretion and degradation, result in the deposition of ECM proteins in the mesangium and basement membrane regions, which leads to pathological changes in glomerular morphology, structure and function, and the development of glomerulosclerosis (5,6). At present, various factors have been identified to be important in the development of DN; however, the underlying mechanisms remain unclear.

Betaine, a neutral zwitterionic compound, is a naturally occurring byproduct of sugar beet refinement, which is extracted from molasses. Betaine has been detected in microorganisms, animals and plants, including wheat, spinach, shellfish and shrimp (7). This compound serves dual roles in human physiology, functioning as an osmolyte and as a methyl donor in transmethylation. As an osmolyte, in order to maintain fluid balance, betaine can protect cells, enzymes and proteins from environmental stresses, including high salinity and extreme temperatures. As a methyl donor, betaine is involved in the methionine cycle in the kidneys and liver in humans (8). In addition, betaine participates in a variety of biological processes. Betaine was reported to suppress prostaglandin synthesis in rat liver macrophages, thus modulating tumor necrosis factor- α secretion and reversing the inhibitory effects of acetaldehyde on the interferon signaling pathway (9,10). Additionally, as a natural food additive, betaine can induce an autoimmune response to regulate the fat:lean mass ratio and the neuro-endocrine system (11).

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Patients with inflammatory bowel disease exhibit notable declines of betaine in urine, which suggests that betaine may be involved in the modulation of immune responses (12). Furthermore, it has been shown that betaine decreased serum glucose and renal oxidative stress in diabetic rats (13). Thus, we speculated that betaine may be an effective agent for the treatment of diabetes and its associated complications. The present study aimed to investigate the effects of betaine on the development of DN, and to determine the underlying potential mechanisms.

Materials and methods

Cell culture. Kidneys from mice were removed in a sterile manner in accordance with the guidelines set by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (14). Briefly, 10 mice aged 5–6 weeks old and weighing 18–20 g were purchased from the Experimental Animal Center of Shanxi Medical University. These mice were maintained under standard conditions (temperature 22°C, 12-h light-dark cycle) and given free access to water and a standard diet. The present study was approved by Institutional Animal Care and Use Committee of Tianjin Third Central Hospital. Mouse mesangial cells (MMCs) were extracted from kidneys and cultured as previously described (15). MMCs were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing HG (30 mM D-glucose) or with normal glucose levels (5.5 mM D-glucose), 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), and a 1% penicillin and streptomycin solution (Sigma-Aldrich; Merck KGaA) for 48 h in a humidified incubator with 5% CO₂ at 37°C.

Cell treatment. MMCs were plated at a density of 5×10⁴ cells/well 24 h prior to treatment. Betaine (1, 5 and 10 μM) and 100 mM metformin (Squibb Pharmaceutical Co., Ltd.) were respectively added to the cells and incubated for 48 h at 37°C under HG conditions (30 mM D-glucose). Cells without any treatment were regarded as the normal control group, while cells treated with metformin alone were regarded as the positive control group.

MTT assay. Cell proliferation was determined by an MTT assay. Briefly, cells at a density of 1.0×10⁶ were seeded into a 96-well culture plate. Following various treatment for 48 h at 37°C, cells were incubated in 0.2 mg/ml MTT solution (Amresco LLC) for 4 h at 37°C. Then, dimethyl sulfoxide was added to each well to dissolve the formazan crystals and the optical density at 490 nm was detected using a Synergy™ Multi-Mode Microplate Reader (Bio-Tek Instruments, Inc.).

Cell cycle assay. For cell cycle analysis, cells were harvested after treatment for 48 h at an initial density of 6.0×10⁵ cells/well in 6-well plates, washed with PBS, and then fixed with 70% ethanol at 4°C overnight. Subsequently, MMCs were incubated with RNase A (50 μg/ml; Sigma-Aldrich; Merck KGaA) and propidium iodide (50 μg/ml; Sigma-Aldrich; Merck KGaA) at 4°C for 30 min. Finally, the cell cycle was analyzed with a flow cytometer (FACSCanto II; BD Biosciences) and CellQuest software (BD Biosciences).

Western blot analysis. For western blot analysis, cells were lysed using lysis buffer (Cell Signaling Technology, Inc.). Total protein was extracted from cells and its concentration was measured with a BCA protein assay kit (Thermo Fisher Scientific, Inc.). Samples were subjected to 11% SDS-PAGE and then transferred to polyvinylidene difluoride membranes. The membranes were incubated with primary antibodies (1:1,000) against fibronectin (ab2413, Abcam), type IV collagen (ab6586, Abcam), p21 (cat. no. 2947, Cell Signaling Technology, Inc.), p27 (cat. no. 3686, Cell Signaling Technology, Inc.), phosphorylated (p)-Akt (cat. no. 9614, Cell Signaling Technology, Inc.), Akt (cat. no. 9272, Cell Signaling Technology, Inc.), p-extracellular-signal-regulated kinase (Erk)1/2 (cat. no. 3510, Cell Signaling Technology, Inc.), Erk1/2 (cat. no. 4695, Cell Signaling Technology, Inc.), p-p38 mitogen-activated protein kinase (MAPK; cat. no. 4511, Cell Signaling Technology, Inc.), p38 MAPK (cat. no. 8690, Cell Signaling Technology, Inc.) and GAPDH (cat. no. 5174, Cell Signaling Technology, Inc.) overnight at 4°C after blocking with 5% non-fat milk at room temperature for 2 h. Subsequently, the membranes were incubated with corresponding horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) at room temperature for 2 h. An enhanced chemiluminescence detection system (SuperSignal West Dura Extended Duration Substrate, Pierce; Thermo Fisher Scientific, Inc.) was used to determine protein expression and the Quantity One analysis system version 4.6 (Bio-Rad Laboratories, Inc.) was used for the quantification of protein expression.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from MMCs using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Complementary DNA was synthesized at 37°C for 15 min and 85°C for 5 sec using a PrimeScript RT Reagent kit (Takara Biotechnology Co., Ltd.) and analyzed with a TaqMan Universal PCR Master Mix kit (Thermo Fisher Scientific, Inc.) under the thermocycling conditions: initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec. The following primer pairs were used for PCR amplification: Fibronectin, forward 5'-GCAGTGACCACCATTTCCTG-3', reverse, 5'-GGT AGCCAGTGAGCTGAACAC-3'; type IV collagen, forward 5'-TCCTTGTGACCAGGCATAGT-3', reverse, 5'-TTGAAC ATCTCGCTCCTCTC-3'; and GAPDH, forward: 5'-ATC CCATCACCATCTTCCAG-3', reverse, 5'-CCATCACGCACA GTTTC-3'. GAPDH was used as an internal control. For relative gene expression quantification, the 2^{-ΔΔC_q} method was employed (16).

Statistical analysis. All experiments were repeated three times. Data were expressed as the mean ± standard deviation. SPSS 17.0 statistical software (SPSS, Inc.) was used for all statistical analyses. One-way analysis of variance followed by a Tukey's test was used for comparisons between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Betaine inhibits the proliferative ability of MMCs via G1-phase arrest. The effects of betaine on the growth of MMCs was

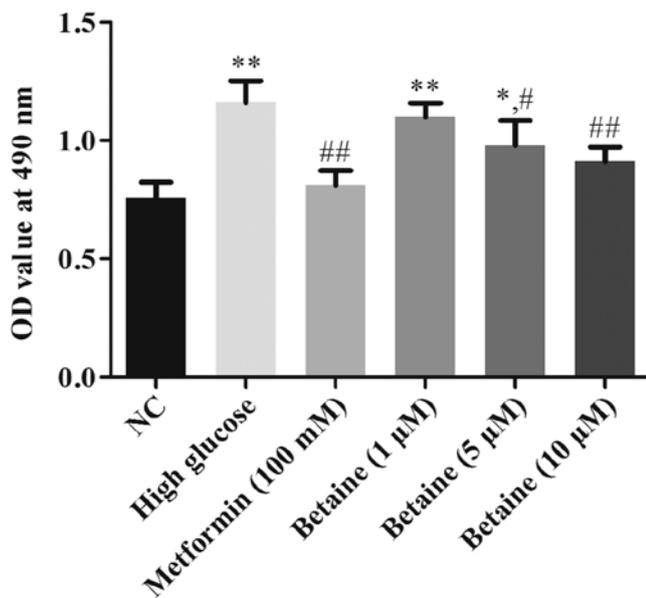


Figure 1. Betaine suppresses the proliferative ability of MMCs. Following treatment, the proliferative ability of MMCs was detected via an MTT assay. Data were expressed as the mean \pm standard deviation. * $P < 0.05$, ** $P < 0.01$ vs. NC; # $P < 0.05$, ## $P < 0.01$ vs. High glucose. MMCs, mouse mesangial cells; NC, negative control; OD, optical density.

determined by an MTT assay. Compared with the control group, the proliferative ability of MMCs was significantly enhanced under HG conditions. Betaine treatment inhibited MMC proliferation in a dose-dependent manner. Metformin significantly repressed MMCs proliferation compared with HG treatment (Fig. 1).

As presented in Fig. 2A, compared with the control group, HG significantly reduced the proportion of cells in G1 phase, while betaine treatment induced G1-phase arrest of MMCs in a dose-dependent manner. Compared with the cells treated with HG, the abundance of G1 phase cells significantly increased in MMCs treated with metformin. In addition, the protein expression levels of p21 and p27 were significantly decreased in MMCs treated with HG compared with control cells, while betaine treatment increased protein p21 and p27 protein expression in MMCs in a dose-dependent manner. Furthermore, a significant increase in the expression of the aforementioned proteins was reported following treatment with metformin compared with the HG conditions (Fig. 2B).

Betaine prevents ECM deposition in MMCs. To investigate the effects of betaine on ECM deposition in MMCs, the expression levels of ECM proteins, including fibronectin and type IV collagen, were determined. As presented in Fig. 3, the protein and mRNA expression levels of fibronectin and type IV collagen were significantly increased in MMCs treated with HG compared with the control cells; betaine treatment decreased the levels of fibronectin and type IV collagen in MMCs in a dose-dependent manner. Additionally, metformin significantly inhibited fibronectin and type IV collagen expression in MMCs compared with the HG conditions (Fig. 3). These results suggested that betaine could prevent ECM deposition induced by HG.

Betaine prevents the activation of Akt, Erk1/2 and p38. To explore the underlying mechanism of the effects of betaine on MMCs, the Akt and MAPK signaling pathways were analyzed. As presented in Fig. 4, the protein expression levels of p-Akt, p-Erk1/2 and p-p38 were significantly increased in MMCs treated with HG compared with control cells. Betaine treatment decreased the levels of p-Akt, p-Erk1/2 and p-p38 in MMCs in a dose-dependent manner. On the contrary, metformin significantly inhibited p-Akt, p-Erk1/2 and p-p38 protein expression in MMCs compared with the HG conditions (Fig. 4). These results indicated that betaine might exert its functions through the Akt and MAPK signaling pathway.

Discussion

In the present study, betaine inhibited cell proliferation, induced G1-phase arrest and reduced ECM deposition in MMCs, possibly via suppression of the Akt/Erk1/2/p38 MAPK signaling pathway. The results revealed that betaine may be a promising therapeutic agent for the treatment of DN.

DN is considered as one of the major microvascular complication of diabetes; ~50% of diabetes cases exhibit DN, which is mainly responsible for end-stage renal disease (17). As DN poses great social and economic burden to individuals, families and society, it is a major public health problem worldwide (18). In China, the proportion of patients with end-stage renal disease caused by DN is increasing every year (19); however, the pathogenesis of DN is markedly complicated and its mechanism has not yet been fully elucidated. As the pathogenesis of DN involves in various bioactive compounds and several signaling pathways, effective preventative and treatment measures are required. Thus, exploring the pathogenesis of DN and identifying potential treatment methods to delay the progression of DN have important social and economic value.

Betaine, a methyl donor, has been reported to possess various physiological and pharmacological functions (20,21). Betaine hydrochloride can be used for the prevention and therapy of atherosclerosis, liver disease gastric acid deficiency and rheumatism (22-27). Betaine possesses notable medicinal value and has broad applications; however, few studies have investigated the effects of betaine on DN. Thus, the current study aimed to investigate the effects and possible mechanism of betaine on HG-induced MMCs. Mesangial cell abnormalities and ECM deposition are pathological hallmarks of DN (6). Various studies have demonstrated that mesangial cell proliferation is crucial in the occurrence and evolvement of DN (6,28). Our findings demonstrated that betaine and metformin inhibited cell proliferation, induced G1-phase arrest and prevented ECM deposition in MMCs.

In addition, the Akt, Erk1/2 and p38 MAPK signaling pathways were determined to be involved in the mechanism underlying the effects of betaine on MMCs. Akt, is a serine/threonine protein kinase reported to be anti-apoptotic and one of the main downstream targets of the phosphatidylinositol (3,4,5)-trisphosphate signaling pathway (29). Inactivation of Akt, a key regulator of cell viability, is involved in degenerative diseases and stress-induced

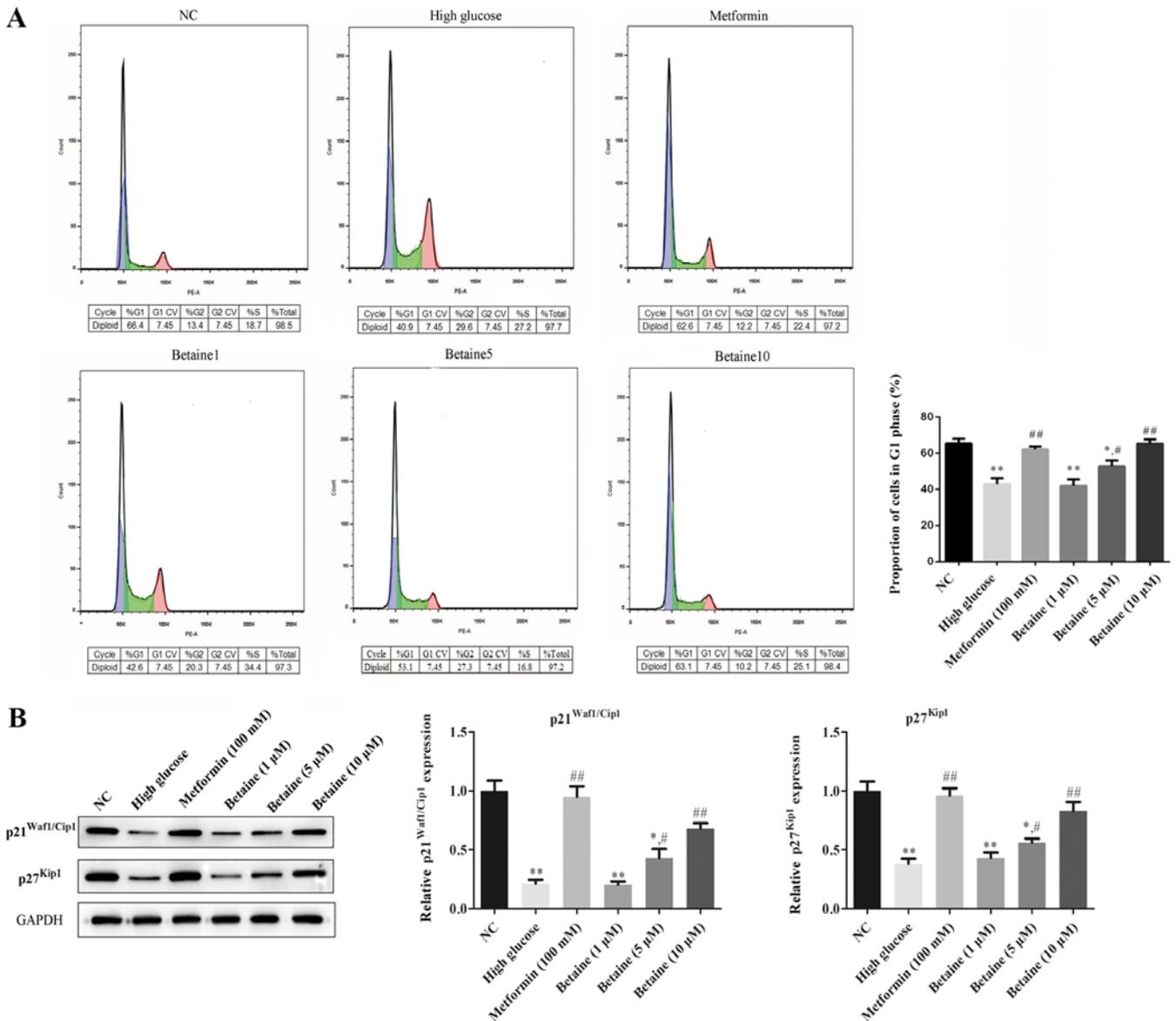


Figure 2. Betaine induces G1 phase arrest of MMCs. (A) Following treatment, the cell cycle distribution of MMCs was detected using flow cytometry and the proportion of cells in G1 phase was quantified. (B) Protein expression levels of p21 and p27 in different groups. Data were expressed as the mean ± standard deviation. *P<0.05, **P<0.01 vs. NC; #P<0.05, ##P<0.01 vs. High glucose. MMCs, mouse mesangial cells; NC, negative control.

pathological cell death (30,31). It has been reported that the Akt signaling pathway is associated with DN (29); an Akt inhibitor was able to attenuate HG-induced cell proliferation, inflammation and ECM expression in mesangial cells (32). Compounds such as daphnetin and zeaxanthin, could ameliorate HG-induced mesangial cell apoptosis via the Akt signaling pathway (32,33). Our results indicated that betaine inhibited MMC proliferation and ECM deposition via the Akt signaling pathway, which is in consistent with previous studies. The Erk1/2 signaling pathway is also involved in DN (34). Erk has been implicated in cell proliferation and differentiation, as it can induce the expression of certain genes (35). As mesangial cell proliferation is facilitated by the activation of Erk1/2, its inhibition protected mesangial cells under HG conditions by suppressing cell proliferation and ECM deposition (36,37). In addition, p38 MAPK, which is associated with cell apoptosis initiation and cell cycle arrest,

has been demonstrated to be activated in glomerular mesangial cells under HG conditions (38,39). In the present study, it was demonstrated that Akt, Erk1/2 and p38 MAPK were activated in MMCs under HG conditions, and betaine was proposed to exert its protective effects via the Akt/Erk/p38 MAPK signaling pathway.

However, there are certain limitations to the present study. There are three isoforms of Akt in mammalian cells, namely Akt1, Akt2 and Akt3. Though it has been reported that Akt2 was strongly associated with the regulation of glucose homeostasis and is the predominant Akt isoform expressed in insulin-responsive tissues (40), the specific binding sites for betaine on Akt were not determined. Additionally, the specific targets activated downstream of the Akt/Erk1/2/p38 MAPK signaling pathway should be investigated in subsequent studies. Furthermore, HG in culture cannot completely mimic diabetic conditions *in vivo*; experiments using diabetic

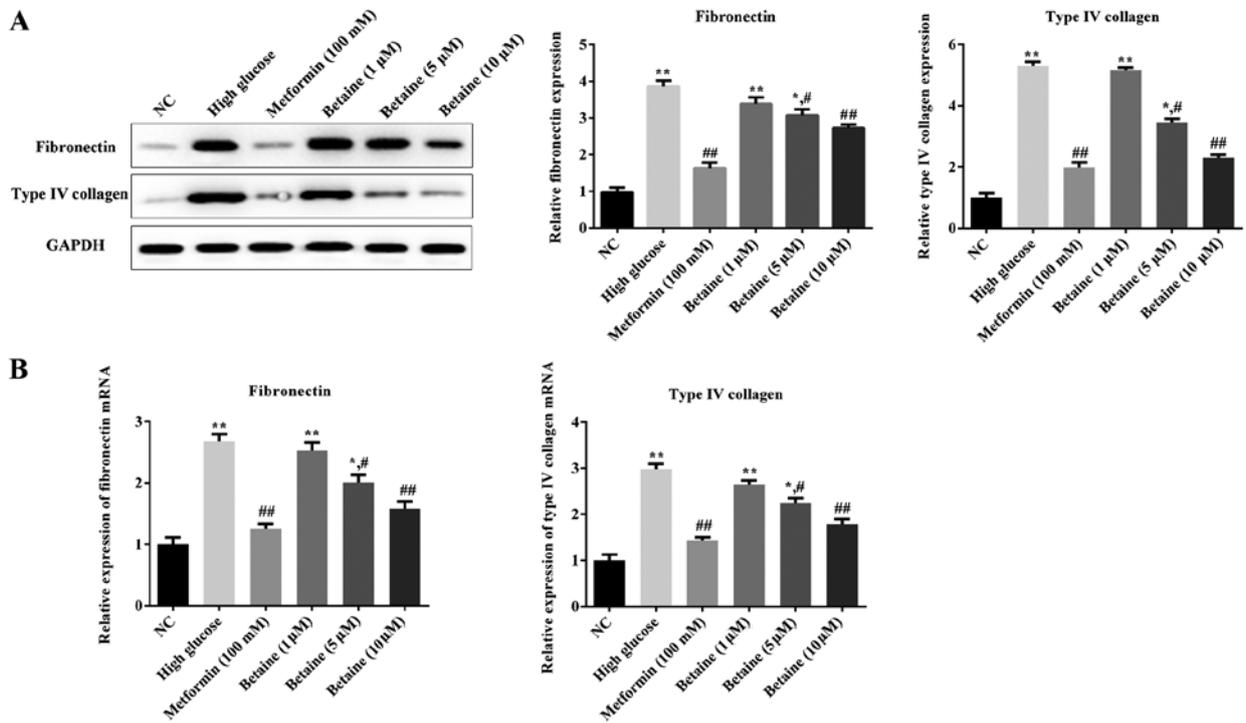


Figure 3. Betaine inhibits fibronectin and type IV collagen expression in mouse mesangial cells. Following treatment, the (A) protein and (B) mRNA expression levels of fibronectin and type IV collagen were detected using western blotting and reverse transcription-quantitative polymerase chain reaction, respectively. *P<0.05, **P<0.01 vs. NC; #P<0.05, ##P<0.01 vs. High glucose. NC, negative control.

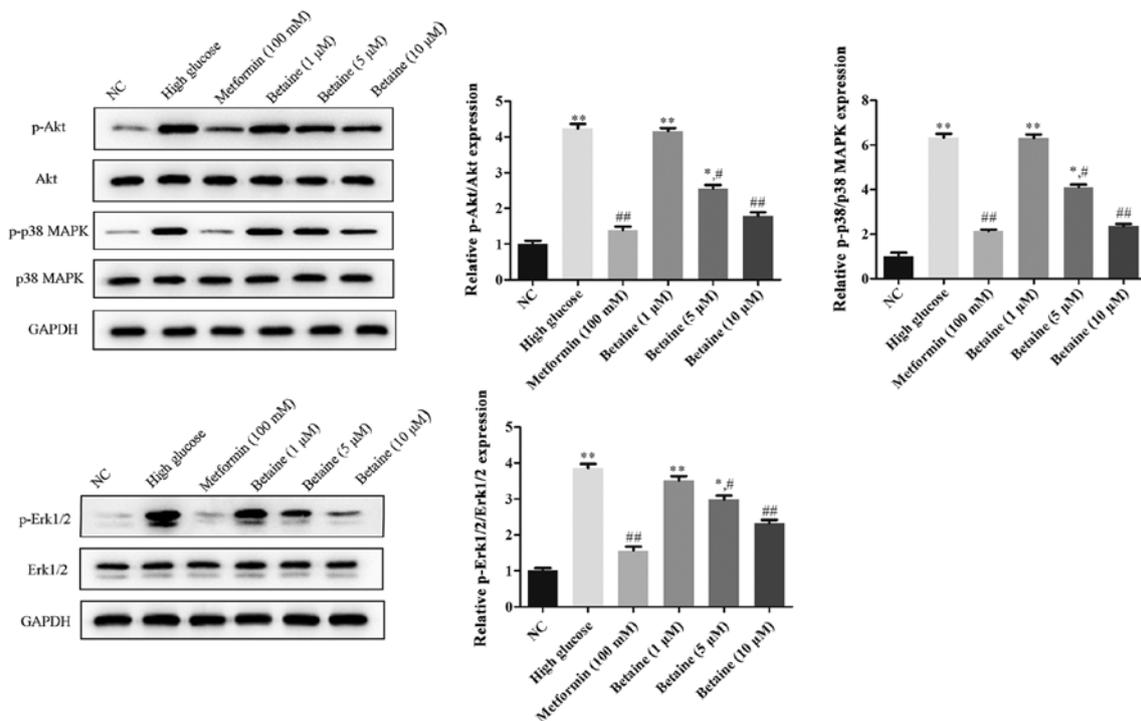


Figure 4. Effects of betaine on the activation of Akt, Erk1/2 and p38 in mouse mesangial cells. Following treatment, the protein expression levels of Akt, Erk1/2, p38 MAPK, p-AKT, p-Erk1/2 and p-p38 MAPK was measured via western blotting. *P<0.05, **P<0.01 vs. NC; #P<0.05, ##P<0.01 vs. High glucose. AKT, protein kinase B; Erk, extracellular-signal-regulated kinase; MAPK, mitogen-activated protein kinase; NC, negative control; p, phosphorylated.

mouse models should be performed to validate these preliminary data. The present study reported the protective effects of betaine *in vitro*; the effects of betaine treatment *in vivo* should be determined in the future.

Collectively, the findings of the current study indicated that betaine exerted a protective effect on MMCs under HG conditions by inhibiting MMC proliferation and ECM deposition via regulation of the Akt/Erk1/2/p38 MAPK signaling pathway.

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Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

XL made substantial contributions to the design of the present study. LW and HM performed the experiments. XL and HM analyzed the data. XL and LW wrote the manuscript. XL revised the manuscript. All authors reviewed the manuscript.

Ethics approval and consent to participate

The present study was approved by Institutional Animal Care and Use Committee of Tianjin Third Central Hospital and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (14).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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