Thalidomide decreases high glucose-induced extracellular matrix protein synthesis in mesangial cells via the AMPK pathway

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Abstract. A previous study demonstrated the renal-protective effect of thalidomide (Thd) in diabetic nephropathy rats through the activation of the adenosine monophosphate-activated protein kinase (AMPK) and inhibition of the nuclear factor κB (NF-κB)/monocyte chemoattractant protein-1 (MCP-1) and transforming growth factor (TGF)-β1/mothers against decapentaplegic homolog signaling pathways. The association between AMPK inactivation and high glucose (HG)-induced meningeal cell (MC) proliferation and extracellular matrix (ECM) accumulation via NF-κB and TGF-β1 signaling remains unknown. The aim of the current study was to demonstrate the effects of Thd on cell proliferation and ECM expression in HG-cultured MCs and the underlying mechanisms. HG-cultured human MCs were treated with Thd. Cell proliferation was measured by MTT assay and quantification of cell proliferation was based on the measurement of bromodeoxyuridine incorporation. The differences in TGF-β1, fibronectin and MCP-1 protein expression levels were detected via ELISA and western blot analysis. The AMPK signaling pathway was also examined by western blot analysis in MCs. Compound C, an AMPK inhibitor and AICAR (5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside), an AMPK agonist, were used to analyze the functional role of AMPK in MCs. Cell proliferation was significantly decreased in HG-cultured MCs following treatment with high concentrations of Thd (100 and 200 µg/ml) for 24 h compared with the HG-cultured MC group. Thd suppressed the inflammatory processes in HG-induced MCs. These effects were partially mediated through the activation of AMPK and inhibition of the NF-κB/MCP-1 signaling pathways. Taken together, these results suggest that Thd may have therapeutic potential in diabetic renal injury via the AMPK signaling pathway.

Introduction

Diabetic nephropathy (DN) is one of the main causes of end-stage renal disease worldwide (1). Typical morphological changes of DN include proliferation of mesangial cells (MCs) and accumulation of the extracellular matrix (ECM), which contributes to the thickening of basement membranes and glomerulosclerosis (2,3). It is therefore important to develop effective therapeutic approaches for the treatment and prevention of glomerulosclerosis in diabetes, which may include inhibition of MC proliferation and ECM accumulation. Hyperglycemia and inflammation serve roles in the initiation of DN (4). Inflammation and pro-inflammatory cytokines are critical to the pathogenesis of DN (5). Nuclear factor κB (NF-κB) is considered to be a major signaling pathway associated with inflammation, regulating several inflammatory response genes including monocyte chemoattractant protein-1 (MCP-1) and transforming growth factor (TGF)β1 (5). MCP-1 and TGF-β1 are secreted by glomerular MCs and are inflammatory mediators in DN (5,6). MCP-1, TGF-β1 and fibronectin (FN) secretion can be enhanced via NF-κB activation, leading to MC proliferation, abnormal ECM accumulation and tubulointerstitial sclerosis (7,8).

Adenosine monophosphate-activated protein kinase (AMPK) is an enzyme associated with cellular energy homeostasis (9). Exposure to high glucose (HG) concentrations suppresses AMPK activation (10-14), inhibiting the activation of NF-κB (15). In addition, inactivation of AMPK is associated with pro-inflammatory and pro-fibrotic damage (16).

Thalidomide (Thd) was previously withdrawn from the market due to the high incidence of teratogenicity (17). However, the return of Thd in clinical practice is primarily due to its anti-inflammatory, anti-fibrotic and immune-modulatory properties, as it is currently used for the treatment of inflammatory bowel disease, multiple myeloma and rheumatic disease (17). Thd can suppress the levels of pro-inflammatory cytokines, which include tumor necrosis factor α, interleukin (IL)-1β, IL-6 and TGF-β1 (17-19). Previous studies have demonstrated the anti-inflammatory effects of Thd in diabetic retinopathy, neuropathy and cardiomyopathy animal models (20-22). A previous study demonstrated the renal-protective effect of Thd...
in DN rats through the activation of AMPK and inhibition of NF-xB/MCP-1 and TGF-β1/mothers against decapentaplegic homolog (Smad) signaling pathways (23). The association between Thd-induced AMPK activation and the inhibition of HG-induced MC proliferation and ECM accumulation via NF-xB and TGF-β1 signaling remains unknown. The aim of the present study was to demonstrate the effects of Thd on cell proliferation and ECM expression in HG-cultured MCs and the underlying mechanisms.

Materials and methods

Cell culture. Human mesangial cell line T-SV40 was donated by Dr Li Xuewang at Union Medical College Hospital (Beijing, China). Cells were cultured in Dulbecco's modified Eagle's media (DMEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences) and maintained at 37˚C in a 5% CO2-humidified incubator. Medium was changed every 2 days and only cells at passage 3-5 were subsequently used. Cells were synchronized prior to use in subsequent experiments following pre-incubation with Minimum Essential Medium (MEM; HyClone; GE Healthcare Life Sciences) supplemented with 1% FBS at 37˚C overnight. Cells were subsequently exposed to Normal glucose (NG) containing 5.6 mmol/l D-glucose and HG containing 30 mmol/l D-glucose with additional administration of Thd (0, 10, 50, 100 and 200 µg/ml) at 37˚C for 12, 24 or 48 h.

Reagents. Thd (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich) and diluted in saline to a final concentration of 100 mg/ml stock solution. In the Thd-treated groups, the Thd stock solution was diluted in DMSO to a final concentration of 0.2%. The control groups received the corresponding volume of vehicle control (DMSO only). AMPK inhibitor (compound C) was purchased from Merck KGaA and AMPK agonist (5-aminomimidazole-4-carboxamide 1β-D-ribofuranoside; AICAR) was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). For compound C (C) or AICAR (A) treatment, synchronized MCs were treated with or without 10 µM C or 1 mM A under NG at 37˚C for 1 h. Following inhibitor or activator incubation, cells were treated under HG with or without Thd (100 µg/ml) at 37˚C for 24 h.

MTT assay. Cell proliferation was measured by MTT assay. Following 12, 24 or 48-h treatment with various concentrations (0, 10, 50, 100 and 200 µg/ml) of Thd, MCs were seeded into 96-well plates, as described above. The cells were subsequently treated with BrdU during the final 2 h of incubation. The BrdU Cell Proliferation assay kit (cat. no. 2750; EMD Millipore, Billerica, MA, USA) was performed according to the manufacturer's protocol.

ELISA. Cell supernatant was harvested from the NG group and different treatment groups under HG with Thd (0, 10, 50, 100 and 200 µg/ml) and centrifuged at 1,500 x g for 10 min at 4˚C. The level of TGF-β1 (cat. no. EK0513), MCP-1 (cat. no. EK0441) and FN (cat. no. EK0349) secreted were detected using ELISA kits (all Boster Biological Engineering Co., Wuhan, China) (24). Absorbance was measured at 450 nm using a microplate reader (Model 550; Bio-Rad Laboratories, Inc.). Each sample was analyzed in triplicate.

Western blot analysis. MCs were washed with PBS. Total protein was extracted from MCs using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) supplemented with protease (cat. no. HY-K0011) and phosphatase inhibitors (cat. no. HY-K0021; each MedChemExpress, Monmouth Junction, NJ, USA), which were added prior to use. Total protein was quantified using a bicinchoninic acid assay (Beyotime Institute of Biotechnology) and 50 µg protein/lane was separated via SDS-PAGE on a 12% gel. The separated proteins were subsequently transferred onto nitrocellulose membranes (Whatman International Ltd., Maidstone, Kent, UK) and blocked using blocking buffer (PBS and 0.1% Tween-20) with 5% non-fat milk for 1 h at room temperature. Then membranes were incubated with primary antibodies against TGF-β1 (1:1,000; cat. no. ab92486), MCP-1 (1:1,000; cat. no. ab9669), inhibitor of NF-xB (IxBα) (1:1,000; cat. no. ab32518), β-actin (1:1,000; cat. no. ab8226; all Abcam, Cambridge, UK), NF-xB (1:1,000; cat. no. 4764), AMPK (1:1,000; cat. no. 2532), phosphorylated AMPK (p-AMPK; 1:1,000; cat. no. 50081), tubulin (1:1,000; cat. no. 2148) and GAPDH (1:1,000; cat. no. 5174; all Abcam) overnight at 4˚C. Membranes were washed three times with Tris-buffered saline containing Tween®-20. Membranes were incubated with corresponding horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (1:5,000; cat. no. sc2370) or anti-mouse IgG (1:2,000; cat. no. sc2380) secondary antibodies (each, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 90 min at room temperature. The membranes were then reacted with an ECL-plus chemiluminescent detection HRP reagent (Beyotime Institute of Biotechnology). Protein expression was quantified using the Quantity One analysis system (version 4.62; Bio-Rad Laboratories, Inc.).

Statistical analysis. Data are presented as the mean ± standard error of the mean. All statistical analyses were performed using SPSS software (version 19.0; IBM Corp., Armonk, NY, USA). Differences between groups were measured using a one-way analysis of variance followed by a post hoc Bonferroni correction test. P<0.05 was considered to indicate a statistically significant difference.
Results

In vitro dosage of Thd in human MCs. To determine the in vitro dosage of Thd in MCs, cell viability was measured by MTT assay. The MTT assay was performed following treatment with various concentrations (0, 10, 50, 100 and 200 µg/ml) of Thd for 12, 24 or 48 h. Cell viability significantly increased in HG-cultured MCs compared with NG-cultured MCs for 12, 24 and 48 h (Fig. 1). Cell viability significantly decreased in HG-cultured MCs following treatment with Thd 100 and 200 µg/ml for 24 h compared with the HG-cultured MC group. In addition, similar results were observed in HG-cultured MCs following treatment with Thd for 48 h. There was no significant cytotoxic effect observed in HG-induced MCs following treatment with Thd for 12 h.

BrdU assays were performed to examine the effects of Thd on MC cell proliferation. BrdU incorporation significantly increased in HG-cultured MCs compared with NG-cultured MCs for 12, 24 and 48 h (Fig. 1). Cell viability significantly decreased in HG-cultured MCs following treatment with Thd 100 and 200 µg/ml for 24 h compared with the HG-cultured MC group. In addition, similar results were observed in HG-cultured MCs following treatment with Thd for 48 h. There was no significant cytotoxic effect observed in HG-induced MCs following treatment with Thd for 12 h.

Figure 1. Effects of Thd on HG-induced MC viability by MTT assay. NG-cultured MCs were grown in DMEM containing 5.6 mmol/l D-glucose, whilst HG-cultured MCs were grown in Dulbecco's modified Eagle's medium containing 30 mmol/l D-glucose and following treatment with various concentrations (0, 10, 50, 100 and 200 µg/ml) of Thd for 12, 24 or 48 h the MTT assay was used to measure cell viability. Data are presented as the mean ± standard error of the mean (n=5). *P<0.01 and **P<0.001 vs. NG group; #P<0.01 and ##P<0.001 vs. HG group. Thd, thalidomide; HG, high glucose; MC, mesangial cell; NG, normal glucose.

Effect of Thd on TGF-β1, FN and MCP-1 expression. The expression levels of TGF-β1, FN and MCP-1 were analyzed in MCs by ELISA. The protein expression levels of TGF-β1, FN and MCP-1 were significantly enhanced in HG-cultured MCs compared with NG-cultured MCs (Fig. 3). However, the expression levels significantly decreased in HG-cultured MCs following treatment with high concentrations of Thd (100 and 200 µg/ml) compared with the HG-cultured MC group.

Thd modulates the expression of NF-κB, IκBα, MCP-1 and TGF-β1 in HG-induced MCs. To investigate the potential involvement of the NF-κB/MCP-1 signaling pathways in the regulation of HG-induced cell proliferation, the protein expression levels of NF-κB, IκBα, MCP-1 and TGF-β1 were determined by western blot analysis (Fig. 4A). The expression levels of inflammatory cytokines NF-κB, MCP-1 and TGF-β1 were significantly increased whereas the expression level of IκBα was significantly decreased in HG-cultured MCs compared with NG-cultured MCs (Fig. 4B). In addition, treatment with Thd significantly decreased the expression levels of HG-induced inflammatory cytokines NF-κB, MCP-1 and TGF-β1 whereas the expression level of IκBα was significantly increased in HG-cultured MCs (Fig. 4B). These results suggest that Thd may exert anti-inflammatory effects on HG-induced MCs.

Thd attenuates HG-induced NF-κB and TGF-β1 through AMPK signaling. The protein expression levels of AMPK and p-AMPK were determined by western blot analysis in MCs (Fig. 5A). The expression level of p-AMPK was significantly decreased in HG-cultured MCs compared with NG-cultured MCs (Fig. 5B).
Figure 2. Effects of Thd on HG-induced MC proliferation by BrdU assay. NG-cultured MCs were grown in DMEM containing 5.6 mmol/l D-glucose, whilst HG-cultured MCs were grown in DMEM containing 30 mmol/l D-glucose and following treatment with various concentrations (0, 10, 50, 100 and 200 µg/ml) of Thd for 12, 24 or 48 h the BrdU assay was used to quantify cell proliferation. Data are presented as the mean ± standard error of the mean (n=5). *P<0.01 and **P<0.001 vs. NG group; #P<0.01 and ##P<0.001 vs. HG group. Thd, thalidomide; HG, high glucose; MC, mesangial cell; BrdU, bromodeoxyuridine; DMEM, Dulbecco's modified Eagle's medium; NG, normal glucose.

Figure 3. TGF-β1, FN and MCP-1 protein expression in MC supernatant. Following treatment with various concentrations (0, 10, 50, 100 and 200 µg/ml) of Thd for 24 h, the protein expression levels of TGF-β1, FN and MCP-1 were analyzed by ELISA. Data are presented as the mean ± standard error of the mean (n=3). *P<0.01 and **P<0.001 vs. NG group; #P<0.01 and ##P<0.001 vs. HG group. TGF-β1, transforming growth factor β1; FN, fibronectin; MCP-1, monocyte chemoattractant protein-1; MC, mesangial cell; Thd, thalidomide; NG, normal glucose; HG, high glucose.
In addition, treatment with high concentrations of Thd (100 and 200 µg/ml) significantly increased the expression level of p-AMPK (Thr172) compared with the HG-cultured MC group (Fig. 5B). Furthermore, treatment with Thd appeared to activate AMPK and inhibit TGF-β1 and NF-κB protein expression in a similar manner to AICAR. By contrast, compound C appeared
to reverse Thd-induced AMPK activation and enhance TGF-β1 and NF-κB protein expression (Figs. 6 and 7).

Effects of the AMPK signaling pathway on cell viability and the cytotoxic potential of Thd. In order to examine the effects of the AMPK signaling pathway in HG-cultured MCs, cell proliferation was measured by MTT assay. Cell viability was significantly decreased in HG-cultured MCs following treatment with Thd 100 µg/ml compared with the HG-cultured MC group. Similarly, cell viability significantly decreased in
HG-cultured MCs following treatment with AICAR for 12 or 24 h compared with the HG-cultured MC group. However, the Thd-induced decrease in cell viability was significantly reversed in HG-cultured MCs following treatment with compound C (Fig. 8A). In addition, there was no significant cytotoxic effect observed in NG-induced MCs following treatment with Thd (100 µg/ml) for 12, 24 or 48 h (Fig. 8B).

Discussion

The current study is the first to identify the protective effects of Thd against HG-induced matrix protein synthesis in human mesangial cells, to the best of our knowledge. Following 24-h Thd incubation, HG-induced inflammatory cytokines and abnormal ECM protein accumulation were significantly decreased compared with the HG group. These protective effects may be due to the upregulation of p-AMPK and inhibition of the NF-κB/MCP-1 signaling pathways.

Chronic inflammation serves a role in the initiation of DN (4,5). Several inflammatory cytokines including adhesion molecules and pro-inflammatory cytokines, are thought to be involved in the pathogenesis of DN (4,25). NF-κB, a major signaling pathway in inflammation, is activated in response to cellular stress which includes hyperglycemia, obesity and oxidative stress (26). NF-κB activation induces the transcription of several target genes including MCP-1, TNF-α and the interleukin system (26). MCP-1 can accelerate the recruitment of macrophages to the kidneys following renal injury in DN, whereas TGF-β1 upregulation can inhibit the accumulation of ECM in DN (27-29). In the current study, the expression levels of the inflammatory cytokines NF-κB, MCP-1 and TGF-β1 significantly increased in HG-induced MCs, and significantly decreased following treatment with Thd. By contrast, treatment with Thd significantly increased the expression level of IκBα. These results suggest that Thd may exert anti-inflammatory effects through inhibition of the NF-κB signaling pathway.

Previous studies have demonstrated that AMPK activation in the diabetic kidney can be inhibited via multiple mechanisms (30,10,11). Studies have revealed that the down-regulation of p-AMPK aggravates the abnormal accumulation of ECM proteins and glomerular hypertrophy via activation of the mammalian target of rapamycin and the TGF-β1/Smad4 signaling pathway (30,31). AMPK activation serves a role in the downregulation of TGF-β1 in DN (32). Previous studies have demonstrated that AMPK activation can inhibit the activation of NF-κB (12,13). AMPK could therefore be used as a target for the treatment of DN. In the present study, treatment with high concentrations of Thd (100 and 200 µg/ml) for 24 h significantly increased the expression level of p-AMPK (Thr172) and significantly decreased the expression levels of inflammatory cytokines NF-κB, MCP-1 and TGFβ compared with the HG-cultured MC group. Furthermore, treatment with Thd appeared to activate AMPK and inhibit TGF-β1 and NF-κB protein expression in a similar manner to AICAR. Incubation with compound C appeared to reverse Thd-induced AMPK activation and enhance NF-κB, MCP-1 and TGF-β1 protein expression. Taken together, these results suggest that the anti-inflammatory effects of Thd depend on AMPK signaling. However, further studies are required to demonstrate the mechanisms of Thd in the AMPK signaling pathway.

In conclusion, the current study is the first in vitro study to demonstrate that Thd reduces inflammation via the activation of AMPK, and the inhibition of the NF-κB/MCP-1 signaling pathway in HG-induced MCs, to the best of our knowledge. These results suggest that Thd may have therapeutic potential in diabetic renal injury. However, further studies are required to elucidate the molecular mechanism underlying Thd-induced AMPK activation in DN.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.
Authors' contributions

HXZ and RSL designed the study and provided research funding. JY and YFL designed the experiments and provided technical guidance. HXZ and JY performed the experiments. HXZ and JY wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

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Patient consent for publication

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Competing interests

The authors declare that they have no competing interests.

References