Janus Kinase 2/Signal Transducers and Activators of Transcription Signal Inhibition Regulates Protective Effects of Probucol on Mesangial Cells Treated with High Glucose

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Received November 15, 2009; accepted January 28, 2010; published online February 2, 2010

Probucol is a cholesterol-lowering drug with an anti-proliferative effect. Excessive growth of glomerular mesangial cells and overexpression of transforming growth factor-β1 (TGF-β1) and connective tissue growth factor (CTGF) are the pathological features of diabetic nephropathy. In this study, human mesangial cells (HMCs) treated with high glucose showed the above-mentioned features through the activation of Janus kinase 2 (JAK2)/signal transducers and activators of transcription (STAT) pathway. Probucol can suppress cell proliferation, down-regulate mRNA and protein levels of TGF-β1 and CTGF in HMCs treated with high glucose. Phosphorylation of JAK2, STAT1 and STAT3 caused by high glucose was obviously prevented in HMCs pretreated with probucol, indicating that the protective effect of probucol on HMCs might be through the inhibition of JAK2/STAT pathway. Therefore, probucol could be a potential therapeutic agent for diabetic nephropathy, and this paper provides new insights into the molecular mechanisms underlying probucol's effects.

Key words mesangial cell; probucol; Janus kinase 2

High glucose activates intracellular signaling processes, including the polyol pathway and the generation of reactive oxygen species (ROS). These pathways further activate the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway in glomerular mesangial cells. The JAK/STAT pathway responds to intracellular ROS, oxidative stress may be a major contributor to diabetes and diabetic nephropathy. The activation of JAK/STAT pathway is essential for high glucose-induced glomerular mesangial cell growth. It also contributes greatly to the production of the cytokine transforming growth factor-β1 (TGF-β1) and extracellular matrix proteins, which contribute to diabetic nephropathy.

Probucol is a drug used clinically for lowering cholesterol with anti-oxidant and anti-proliferative effects. Also, it is a potential inhibitor of DNA damage. Recent studies suggest that probucol may be used for the prevention of type-2 diabetes by preventing the increases in aldose reductase, and partly arrested proteinuria and disease progression in glomerulonephritis in rats. It also exerted multiple beneficial morphological effects, including reduced cardiac fibrosis and cardiac apoptosis in rat models.

Glomerular mesangial cells have a central role in maintaining the structure and function of the glomerular capillary tuft. High glucose-induced growth of glomerular mesangial cells is a characteristic feature of diabetes-induced renal complications. Mesangial cells cultured under high glucose conditions produce TGF-β1, connective tissue growth factor (CTGF) and extracellular matrix molecules at a significantly faster rate than those cultured under normal glucose conditions, which is one of the basic underlying mechanisms of diabetic nephropathy and induce persistent fibrosis in vivo.

To date, there have been no reports on the effect of probucol on the JAK/STAT pathway, the growth of human mesangial cells (HMCs), and the production of TGF-β1 and CTGF in HMCs. In this study, we investigated the effect of probucol on HMCs cultured under high glucose conditions, and also investigated the molecular mechanisms underlying this effect.

MATERIALS AND METHODS

Cell Line and Reagents The human mesangial cells (Cat. No. 4200) and mesangial cell medium (Cat. No. 4201) were purchased from ScienCell Research Laboratories (San Diego, CA, U.S.A.). RPMI Medium 1640 was purchased from Gibco Invitrogen Corp. (Carlsbad, CA, U.S.A.). The antibodies against JAK2 (Cat. No. 3229), P-JAK2 Tyr221 (Cat. No. 3774), STAT1 (Cat. No. 9175), P-STAT1 Tyr701 (Cat. No. 9167), STAT3 (Cat. No. 9132), P-STAT3 Tyr705 (Cat. No. 9131) and β-actin (Cat. No. 4970) were all purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). The antibodies against CTGF (Cat. No. sc-25440) and TGF-β1 (Cat. No. sc-146) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The reverse transcription-polymerase chain reaction (RT-PCR) system (Cat. No. D2639A and DR100A) was purchased from Takara Biotechnology (Dalian, China-subsidiary of Japan TaKaRa Bio Inc.). All other chemicals were purchased from Sigma Aldrich (St. Louis, MO, U.S.A.).

Cell Culture Primary HMCs were seeded in 25-cm² tissue culture flasks in Mesangial Cell Medium under normal glucose (NG, 5.5 mmol/l glucose), high glucose (HG, 25 mmol/l glucose), or control (5.5 mmol/l glucose + 19.5 mmol/l mannitol) conditions. The culture medium was supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin in a 5% CO₂ atmosphere. The cell medium was changed every other day until the cells became confluent. Cells in passages 3—6 were used. The HMCs at about 70—80% confluence, were cultured in serum-free 1640 with NG for 24 h to synchronize the cell

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growth. After that, the media were replaced with fresh serum-free media containing NG, NG plus 19.5 mmol/l mannitol, HG or HG in the presence of different levels of probucol.

**Cell Proliferation Measurements** To examine the anti-proliferative effects of probucol, cell proliferation was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumumbromide (MTT) assays, 7 groups were given different levels of probucol and glucose, seeded at a density of 2000 to 5000 cells/well in a 96-well flat-bottomed microplate. Medium (180 μl) was added to every well. After 44 h, 20 μl 5 mg/ml MTT was added to every well, incubated for 4 h, then the medium was replaced with 200 μl dimethyl sulfoxide, and the 96-well microplate was shaken gently. Next, the absorbance was measured at 570 nm, and these data were transformed into a variable representing the number of cells by using a curve that correlated the absorbance with number of HMCs.

**RNA Extraction and RT-PCR Analysis for TGF-β1 mRNA and CTGF mRNA** Two micrograms of template were reverse transcribed using oligo(dT)18 primers in a final volume of 20 μl. Human TGF-β1 and β-actin were amplified using the following primers: TGF-β1, forward 5'-GGTGGGGCACCAACTATTGC-3', reverse 5'-AGGCTCCAAATTGGGGCAGG-3', yielding a 161-bp PCR product; β-actin, forward 5'-GGTGGGGCGCCCAGGCCACCA-3', reverse 5'-CTCCTTAATGTCACGCACGATTTC-3', yielding a 539-bp PCR product. Reaction conditions were 95 °C for 2 min, 35 cycles at 94 °C for 30 s, 57 °C for 40 s, and 72 °C for 1.5 min, followed by final extension for 10 min at 72 °C, yielding a 379-bp PCR product. The PCR products were subjected to 2% agarose gel electrophoresis and analyzed with a GDS-8000 Bioimaging system (UVP, upland, CA, U.S.A.) and GelWorks 1D grab it software. RNA expression was quantified by comparison with internal-control β-actin.

**Western Blot Studies of JAK2/STAT, TGF-β1 and CTGF Proteins** We investigated the effect of probucol on the JAK2/STAT, including the concentration of probucol and glucose, seeded at a density of 2000 to 5000 cells/well in a 96-well flat-bottomed microplate. Medium (180 μl) was added to every well. After 44 h, 20 μl 5 mg/ml MTT was added to every well, incubated for 4 h, then the medium was replaced with 200 μl dimethyl sulfoxide, and the 96-well microplate was shaken gently. Next, the absorbance was measured at 570 nm, and these data were transformed into a variable representing the number of cells by using a curve that correlated the absorbance with number of HMCs.

**RESULTS**

**Anti-proliferative Effect of Probucol on HMCs** As shown in Fig. 1, the proliferation of HMCs peaked in the HG group, and it was far greater than the proliferation in the NG group (NG: 0.254±0.028 vs. HG: 0.298±0.007, p<0.05, n=3), but no effects of hyperosmolarity were seen in the control group (NG: 0.254±0.028 vs. NG+19.5 mmol/l mannitol: 0.261±0.024, p>0.05, n=3). The proliferation decreased with the rising level of probucol. In the HG+50 μmol/l probucol group, the proliferation was decreased significantly (HG+50 μmol/l probucol: 0.261±0.012 vs. HG: 0.298±0.007, p<0.05, n=3). In the HG+75 μmol/l probucol and HG+100 μmol/l probucol groups, the proliferation of HMCs were over-inhibited (HG+75 μmol/l probucol: 0.207±0.014 vs. NG: 0.254±0.028; HG+100 μmol/l probucol: 0.168±0.028 vs. NG: 0.254±0.028). This result suggested that probucol inhibited the proliferation of HMCs induced by HG in a dose-dependent manner.

**Probucol Reduces the Syntheses of TGF-β1 mRNA and CTGF mRNA** To investigate HMC’s ability to excrete cytokine, mRNA levels of TGF-β1 and CTGF in HMCs were

![Fig. 1. Effect of Probucol on Proliferation Induced by HG](image-url)
evaluated by RT-PCR analysis. As shown in Fig. 2A, HMCs in the HG group showed a significantly higher mRNA levels of TGF-β1 and CTGF than those in the NG group (TGF-β1 mRNA NG: 0.525±0.03 vs. HG: 0.792±0.09, p<0.01, n=3) (CTGF mRNA NG: 0.487±0.11 vs. HG: 0.808±0.12, p<0.01, n=3). However, no significant effects of hyperosmolality were seen on the syntheses of TGF-β1 mRNA and CTGF mRNA. As shown in Figs. 2B and C, the probucol concentrations between 25 to 75 μmol/l all decreased the up-regulation of mRNA levels of TGF-β1 (HG: 0.681±0.023 vs. HG+25 μmol/l probucol: 0.460±0.028; HG+50 μmol/l probucol: 0.497±0.022; HG+75 μmol/l probucol: 0.533±0.021, p<0.01, n=3), and also significantly decreased the up-regulation of mRNA levels of CTGF (HG: 0.658±0.105 vs. HG+25 μmol/l probucol: 0.444±0.101, p<0.01, n=3; HG: 0.658±0.105 vs. HG+50 μmol/l probucol: 0.549±0.08; HG+75 μmol/l probucol: 0.527±0.102, p<0.05, n=3), but the concentration of 25 μmol/l was the most effective.

**Probucol Inhibits HG-Induced Tyrosine Phosphorylation of JAK2, STAT1 and STAT3** To explore the possible mechanism for the protective effect of probucol on HMCs, we investigated the effect of probucol on JAK2/STAT phosphorylation in HMCs. In Fig. 3A, we found that exposure of cells to HG caused increased phosphorylation of JAK2, STAT1 and STAT3. However, they were both decreased with 25 μmol/l and 50 μmol/l probucol, but 50 μmol/l probucol was more effective. In addition, as indicated in Fig. 3B, regardless of the amount of stimulation time of HMCs, 50 μmol/l probucol was effective in decreasing the phosphorylation of JAK2/STAT. Total protein levels of JAK2, STAT1 and CTGF Induced by HG in HMCs. Probucol down-regulated the mRNA levels in HMCs compared to the HG group (#p<0.01). No significant differences were seen between the NG group and the control (NG+19.5 mmol/l mannitol) group. (B) Representative RT-PCR results show probucol inhibited the up-regulation of mRNA levels of TGF-β1 induced by HG in HMCs. Probucol down-regulated the mRNA levels in HMCs compared to the HG group (∗p<0.05) (∗∗p<0.01). Probucol was most effective when the concentration was 25 μmol/l. Densitometry results were based on three experiments.

**DISCUSSION**

Our study focused on JAK2/STAT pathway and two as-
peptides of HMC, cell proliferation and cytokine excretion, which are important in diabetic nephropathy. Several recent studies have provided evidence that probucol has anti-atherosclerotic and anti-diabetic effects, but the effect of probucol on HMCs has not been demonstrated clearly. In our study, we observed that HMCs incubated in HG exhibit abnormal growth and can serve as an in vitro model of diabetic glomerulosclerosis and all of our investigations were aimed at this HMC model.

Recent reports have showed that probucol has anti-proliferative effects on intimal cells and rat mesangial cells. We found probucol inhibited the proliferation of HMCs induced by HG in a dose-dependent manner. As TGF-β1 and CTGF are key cytokines in diabetic nephropathy and other diseases involving fibrosis, we studied TGF-β1 and CTGF. There have been many reports about the anti-fibrotic effect of probucol, our study showed that probucol reduced the up-regulation of mRNA and protein levels of TGF-β1 and CTGF induced by HG. However, there are interesting differences, probucol decreased the HMC proliferation and the synthesis of TGF-β1 and CTGF in a manner independent of doses and 25 μmol/1 of probucol was most effective.

The sensitivity of MTT, Western blot and RT-PCR are different, and probucol may play roles in both before and after synthesis of mRNA, for example, as what is established is probucol is a potential inhibitor of DNA damage, which may have effects on DNA or RNA synthesis, these effects could be covered by the following effects on the protein modification process. The molecular mechanisms for the protective effects of probucol on mesangial cells under high glucose conditions are not absolutely clear, but these observations suggest that probucol may be effective in controlling HMCs proliferation and the fibrotic cytokines which are vital in diabetic nephropathy.

It has been well established that JAK/STAT pathway plays an important role in the proliferation of cells and in the production of TGF-β1 in diabetic nephropathy in vitro and in vivo. We also confirmed this in our present investigation. However, we found that the increased phosphorylation of JAK2, STAT1 and STAT3 induced by HG was attenuated in all of the different combinations of probucol concentrations and exposure times. The proliferation of HMCs, mRNA and protein levels of TGF-β1 and CTGF were all changed with the change in JAK2/STAT. Values are expressed as means ± S.E. for three independent experiments.

Fig. 3. Effects of Probucol on HG-Induced Tyrosine Phosphorylation of JAK2, STAT1 and STAT3
(A) Western blot showed the effects of 25 μmol/l and 50 μmol/l probucol on the JAK2, STAT1 and STAT3 and tyrosine phosphorylation of JAK2, STAT1 and STAT3. (B) Effects of 50 μmol/l probucol at different times. Results shown are representative of three separate experiments.

Fig. 4. Suppression by Probucol of HG-Induced Synthesis of TGF-β1 and CTGF Proteins
HMCs were pretreated with NG, NG+19.5 mmol/l mannitol, HG, HG+25 μmol/l probucol or HG+50 μmol/l probucol for 48 h. Immunoblotting of cell extracts was then performed for TGF-β1 and CTGF as described. No significant differences were found between the control (NG+19.5 mmol/l mannitol) group and NG group, probucol attenuated the overexpression of TGF-β1 and CTGF proteins induced by HG (p<0.05) (t<0.01). TGF-β1 and CTGF were changed by probucol in the same direction with JAK2/STAT. Values are expressed as means±S.E. for three independent experiments.
in our future studies.

Acknowledgments This study was supported by the National Natural Science Foundation (30700369, to Qiuling FAN) and the Scientific Research Foundation of the First Clinical Hospital of China Medical University. We thank the research institute of First Hospital of China Medical University for valuable suggestions and help.

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