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Atrial Natriuretic Peptide Attenuates High Glucose-Activated Transforming Growth Factor-β, Smad and Collagen Synthesis in Renal Proximal Tubular Cells

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Abstract Atrial natriuretic peptide, besides its role in the regulation of volume homeostasis, has been noted to exert cytoprotective effects in several cell types from hypoxia. The present study was performed to explore the effect of ANP on high glucose-activated transforming growth factor-beta1 (TGF-β1), Smad and collagen synthesis in renal proximal epithelial cells. Cultured NRK-52E cells were divided into five groups: (1) normal glucose (5.5 mM), (2) high glucose (35 mM), (3) D-mannitol (29.5 mM), (4) high glucose plus ANP (10^{-6}–10^{-9} M), and (5) high glucose plus ANP (10^{-6} M) and guanylate cyclase inhibitor LY83583 (10^{-7} M) groups. Messenger RNA levels of TGF-β1, Smad2, and collagens were measured by RT-PCR. ELISA, immunocytochemistry and Western blotting were used to detect protein levels of TGF-β1, Smad2, phospho-Smad 2/3 and collagen type 1. We found high glucose to significantly increase mRNA levels of TGF-β1, Smad 2, collagen types I and III and protein levels of TGF-β1, phospho-Smad 2/3 and collagen type 1, but mannitol did not affect their expression. The addition of ANP significantly attenuated high glucose-enhanced mRNA and protein levels of TGF-β1, Smad and collagens. LY83583 blocked the influence of ANP on high glucose-activated TGF-β1, Smad and collagen synthesis. This is the first study to demonstrate that activation of TGF-β1, Smad and collagen synthesis stimulated by high glucose can also be inhibited by exogenous ANP in renal tubular epithelial cells. J. Cell. Biochem. 103: 1999–2009, 2008.

Key words: ANP; high glucose; TGF-beta1; Smad; collagen

Atrial natriuretic peptide (ANP) has been known to play an important role in the regulation of extracellular fluid volume and blood pressure [de Bold et al., 1981; Brenner et al., 1990]. However, ANP also is so known to be cytoprotective in several cell types, including hepatocytes [Gerbes et al., 1998; Kiemer et al., 2000], cardiomyocytes [Kato et al., 2005; Nishikimi et al., 2006], endothelial cells [Kiemer et al., 2002, 2003; Irwin et al., 2005], vascular smooth muscular cells [Baldini et al., 2005], and macrophage [Tsukagoshi et al., 2001]. ANP protects these cells from hypoxia, ischemia-reperfusion, inflammation, and vasoconstrictors. In cultured renal mesangial and tubular cells, ANP also inhibits hypertrophy, and proliferation caused by angiotensin II [Hannken et al., 2001], cyclosporin A [Polte et al., 2002], and high sodium infusion [Rosón et al., 2006]. Diabetes mellitus, one of the most common chronic diseases in the developed countries, associated with a threefold risk of all cardiovascular diseases and is a major cause of renal failure and blindness [Garcia et al., 1974]. Hyperglycemia is the initial trigger

Chao-Sheng Lo and Zhao-Hong Chen equally contributed to this work.

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of vascular, renal and retinal cell damage associated with diabetes [Sharma and Ziyadeh, 1995; Cooper, 1998]. However, it is not clear whether hyperglycemia-induced cell damage can also be modulated by ANP.

High glucose promotes extracellular matrix (ECM) synthesis by stimulating several growth factors, such as transforming growth factor-β (TGF-β) in a variety of cells [Rocco et al., 1992; Ziyadeh et al., 1994; Nakao et al., 1997]. Similarly, TGF-β stimulates ECM production via several signaling pathways, including TGF-β-activated Smads [Nakao et al., 1997]. Activated Smad2 and Smad3 heteroligomerize with Smad4 form the complex and translocate into the nucleus to regulate extracellular target gene expression [Runyan et al., 2005]. Actually, high glucose is recently shown to stimulate synthesis of collagen at least partially by activating the TGF-β dependent Smad signaling pathway in cultured renal and vascular cells [Li et al., 2003].

In diabetic humans and rats, plasma ANP concentration has been reported to be changed [Bell et al., 1989; Shin et al., 1997, 1999]. In the heart and kidney of diabetic rats, ANP synthesis has been found to be significantly increased, particularly, in ventricles, proximal tubules, distal collect duct and medullary collect ducts [Shin et al., 1997; Lai et al., 2002]. Thus, it is reasonable to hypothesize that the alteration of ANP derived from heart or kidney synthesis might attenuate hyperglycemia-induced renal cell injury. To find out this issue, we would first need to investigate the effect of exogenous ANP on the high glucose-activated intracellular signal pathway in cultured cell experiments. Therefore, in this study, we investigated the effect of ANP on high glucose-activated TGF-β, Smad and collagen synthesis in cultured renal proximal tubular epithelial cells.

MATERIALS AND METHODS

Cell Culture

Rat renal tubular epithelial cell line (NRK-52E; ATCC #CRL-1571) was used in this study. NRK-52E cells were grown in 6-well plastic plates or 8-chamber glass slides (Nunc, Naperville, CT) in normal glucose (i.e., 5.5 mM) Dulbecco's modified Eagle's medium (DMEM, pH 7.45; Invitrogen, CA), supplemented with 5% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin, in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

For stimulation, NRK-52E were grown for 24 h in either normal glucose DMEM plus 29.5 mM D-mannitol or 29.5 mM D-glucose in the absence or presence of guanylate cyclase inhibitor LY-83583 (10^{-7} M) to block cGMP production. Then, cells were harvested for total RNA or protein extraction.

RNA Isolation and Reverse Transcription-Polymerase Chain Analysis

Total RNA was extracted from NRK-52E with Trizol reagent according to manufacturer's directions (Invitrogen) with quantification by its absorbance at 260 nm. Isolated total RNA was used for reverse transcriptase-PCR (RT-PCR) assay to quantify mRNA expression. Briefly, 4 μg of total RNA was used to synthesize first-strand cDNAs by employing the AccessQuick™ RT-PCR System (Promega, Madison, WI). Then, the first strand cDNA was diluted with water to a ratio of 1:4, and the aliquots were processed to amplify rat cDNA fragments with the PCR core kit according to the manufacturer's instructions (Invitrogen). First-strand cDNA (5 μl) and 400 nM primers were added to a final volume of 50 μl PCR mixture (final concentration: 1× PCR buffer, 0.2 mM dNTP, 2 mM $MgCl_2$, and 2.0 units of Taq DNA polymerase) (Invitrogen). After an initial denaturation step at 94°C for 5 min, cDNA fragments were amplified with each amplification profile in a Perkin-Elmer Cetus 9600 thermocycler (Perkin-Elmer Cetus, Norwalk, CT). The primers used in PCR amplification are listed in Table I. To identify cDNA fragments, 10 μl of the PCR products were electrophoresed on 1.2% agarose gel. Gels were photographed by a gel 1000 ultraviolet documentation system (BioRad, Hercules, CA), and analyzed by densitometry. All mRNA levels were normalized by corresponding β-actin mRNA levels.

Western Blot Analysis of p-Smad 2/3, Smad 2/3 and Collagen Type I

NRK-52E cells were grown in 6-well plates with normal glucose (i.e., 5.5 mM), high glucose (i.e., 35 mM), or D-mannitol (i.e., 29.5 mM) for Western blot analysis. Cells were washed with phosophate-buffered saline (PBS) and then the total protein was extracted with PRO-PREP protein extraction solution (iNtRON Biotechnology, Korea). Twenty micrograms of protein from samples were separated on 10% SDS gels

TABLE I. Primer Sequences for PCR

Gene	Sense	Antisense
TGF-β1	5′-tgctaatggtggaccg-3′	5′-acgtcaaaagacagcc-3′
Smad-2	5′-caaggcgatcgagaactgcg-3′	5′-gccgtctacagtgagtgagg-3′
Collagen type I	5′-tgccgtgacctcaagatgtg-3′	5′-cacaagcgtgctgtaggtga-3′
Collagen type III	5′-ctggaccaaaaggtgatgctg-3′	5′-tgccaggggaatcctcgatgtc-3′
β-Actin	5′-cgtaaagacctctatgccaa-3′	5′-agccatgccaaatgtctcat-3′

by SDS–PAGE. Separated protein was transferred onto polyvinylidene difluoride (PVDF) membrane blocked at room temperature for 1 h or overnight at 4°C in Tris-buffered saline with 0.2% Tween 20 (TBS-T) containing 5% skim milk and probed with primary antibodies. Primary antibodies against collagen type I was diluted 1:500 in TBS-T. Anti-Smad 2/3 antibody, which recognizes both phosphorylated and nonphosphorylated Smad 2/3, was diluted 1:250 in TBS-T. Secondary antibodies included horseradish peroxidase (HRP)-labeled anti-rabbit IgG for detection of collagen type I and p-Smad 2/3 (Santa Cruz Biotechnology, Inc., CA) proteins and HRP-labeled anti-goat IgG for detection of Smad 2/3 proteins (Santa Cruz Biotechnology, Inc.). All secondary antibodies were diluted 1:10,000 with TBS-T. The blots were detected by using the ECL visualized by detection kit (Amersham International, UK) according to the manufacturer's instructions.

Immunocytochemistry

NRK-52E cells were cultured in eight-chamber glass slides with normal, high glucose and D-mannitol at 37°C for 24 h. The activation of Smad 2/3 was detected by rabbit polyclonal p-Smad2/3 antibody. Briefly, cells were fixed in 3.7% paraformaldehyde and preincubated with antibody diluent containing background-reducing components (DAKO, CA). Cells were then incubated with the anti-phospho-Smad2/3 antibody or mouse IgG as a positive control at 4°C overnight. After the incubation, peroxidase-conjugated goat anti-rabbit IgG was added to the cells and then the rabbit peroxidase anti-peroxidase complexes. After being washed, slides were developed with diaminobenzidine to produce a brown color, and the cell nucleus was counterstained with hematoxylin.

To quantify the translocation of p-Smad2/3 into nuclei, at least 1,000 cells were counted for the percentage of positive cells with nuclear staining for p-Smad 2/3 under high power (200×) in each well. All scoring was performed blind to the calculator on coded slides.

Ezyme-Linked Immunoassay

The concentrations of TGF-β1 secreted into the medium were measured by an enzyme-linked immunoassay kit (Emax ImmunoAssay system; Promega). Cells were grown in 6-well plates and stimulated with normal glucose, D-mannitol, or high glucose with/without ANP. Medium were collected at 0, 12, 24, and 72 h for TGF-β1 detection. Each experiment was repeated at least six times throughout the study.

Statistical Analyses

Data are expressed as the mean ± SD. Statistical analyses were performed using GraphPad Prism 3.0 (GraphPad Software, Inc., San Diego, CA). Differences were assessed by one-way ANOVA and Bonferroni test.

RESULTS

Effect of Different Concentration of D-Glucose and Mannitol on TGF-β_1 Expression

The expression of TGF-β1 mRNA was increased in a dose-dependent manner in NRK-52E cells (Fig. 1A) by high glucose stimulation. The secretion of TGF-β1 by NRK-52E cells into culture medium was also increased by high glucose in dose-response manner after a 24-h incubation period (Fig. 1B). However, TGF-β1 mRNA and protein levels did not change under different concentration of mannitol in cultured NRK-52E cells.

ANP Attenuated High Glucose-Stimulated TGF-β_1 Synthesis

As shown in Figure 2A, TGF-β_1 mRNA level of NRK-52E cells increased (290%) by 35 mM glucose stimulation as compared with that in the presence of normal glucose concentration at 24 h. In contrast, cells cultured in 29.5 mM mannitol did not increase TGF-β_1 mRNA expression. The increase of TGF-β_1 mRNA by high glucose was reduced in a dose-dependent manner by the treatment of 10^{-8}–10^{-6} M ANP at 24 h. It appeared that the addition of 10^{-6} M ANP

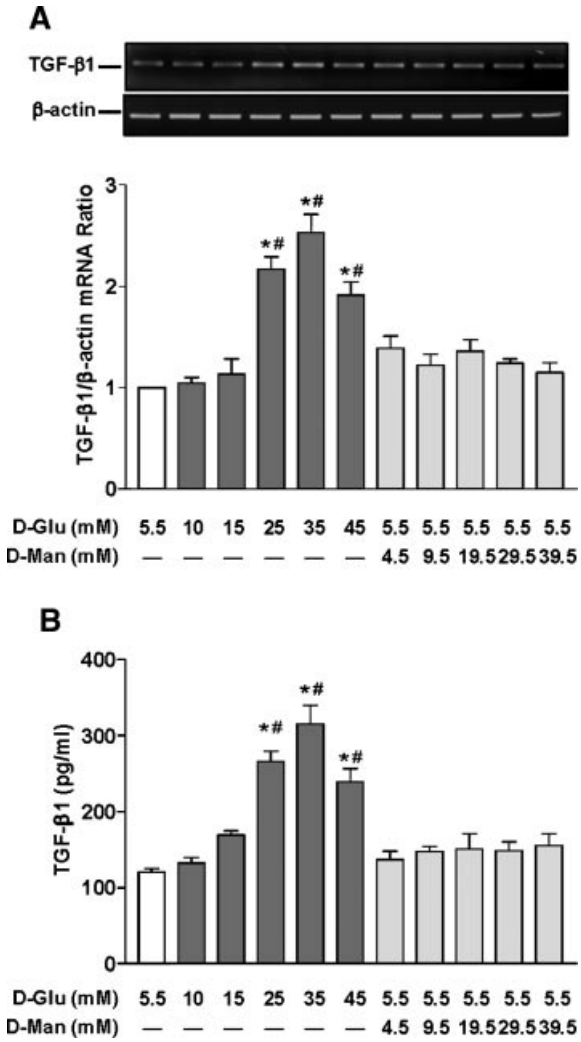

Fig. 1. Effect of different concentrations of D-glucose and D-mannitol on TGF-β_1 mRNA expression in NRK-52E cells. **A**: Cells were incubated for 24 h in the presence of different concentrations of D-glucose (5.5, 15, 25, 35, and 45 mM) and mannitol (4.5, 10, 20, 30, and 40 mM). Cells were collected and assayed for rat TGF-β1 and β-actin mRNA levels by RT-PCR. The relative densities of TGF-β1 were normalized by the β-actin level. TGF-β1 mRNA and β-actin mRNA levels in 5.5 mM glucose were considered as controls. **B**: TGF-β1 and β-actin protein in cultured media were assayed by ELISA. Each point represents the mean ± SD of three independent experiments performed in triplicate. $^*P < 0.05$ versus 5.5 mM glucose; $^{\#}P < 0.05$ versus 4.5–40 mM mannitol.

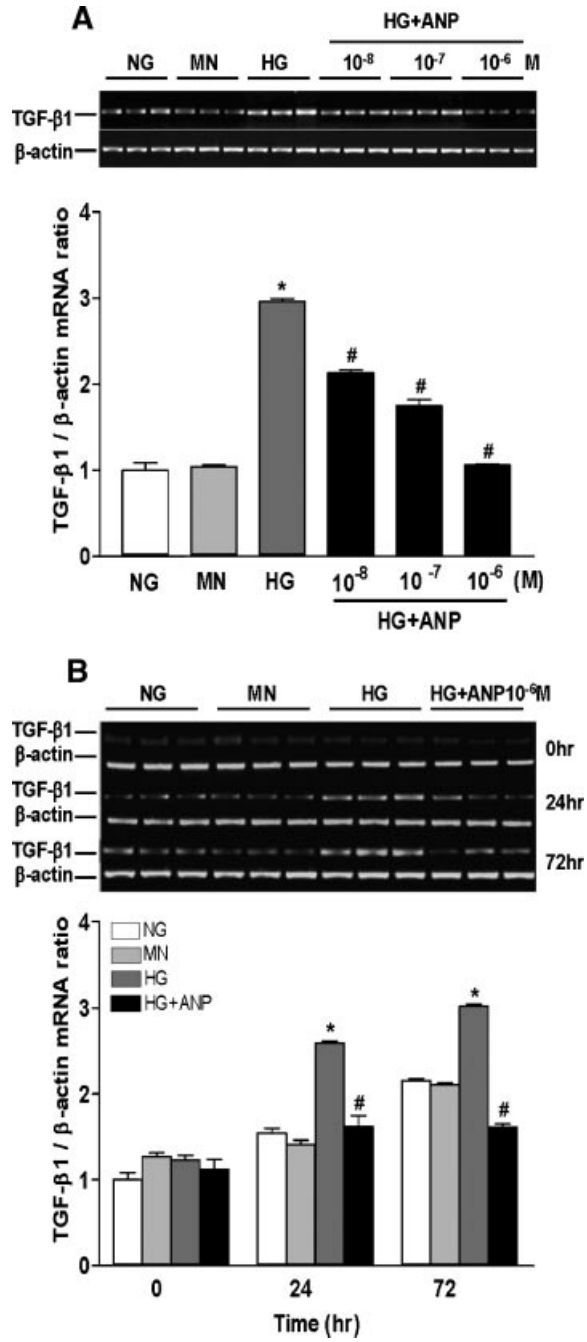

Fig. 2. ANP inhibited high glucose-stimulated overexpression of TGF-β_1 mRNA. **A**: TGF-β1 mRNA levels are assayed by RT-PCR in NRK-52E cells which were incubated in DMEM with normal glucose (NG, 5.5 mM), high D-glucose (HG, 35 mM), D-mannitol (MN, 29.5 mM), or 35 mM D-glucose plus ANP (HG + ANP 10^{-8}–10^{-6} M) for 24 h. **B**: TGF-β1 mRNA levels in NG, MN, HG, and HG + ANP (10^{-6} M) NRK-52E cells, incubated for 0, 24, 72 h. The relative densities of TGF-β1 were normalized with the β-actin control. TGF-β1 mRNA levels in NRK-52E cells incubated in normal glucose medium were considered as controls. Each point represents the mean ± SD of three independent experiments performed in triplicate. $^*P < 0.05$ versus NG or MN; $^{\#}P < 0.05$ versus HG.

reduced TGF-β_1 mRNA to 37.5% of TGF-β_1 mRNA level by 35 mM high glucose stimulation. Figure 2B shows that ANP could effectively inhibit high glucose-stimulated TGF-β_1 mRNA expression after treatment with 10^{-6} M ANP at 24 and 72 h.

In Figure 3A, 35 mM glucose significantly increased TGF-β_1 protein level at 24 h in the

conditioned medium of NRK-52E cells, while 29.5 mM mannitol did not increase TGF-β_1 level. High glucose-increased TGF-β_1 protein levels were significantly reversed in a dose-dependent manner by the treatment of 10^{-9}–10^{-6} M ANP at 24 h. As can be seen by Figure 3B, ANP could effectively inhibit the effect of high glucose on TGF-β_1 level after treatment with 10^{-6} M ANP at 12, 24, and 72 h.

ANP Attenuated High Glucose-Activated Smad 2 mRNA Expression and Protein Phosphorylation

In Figure 4, 35 mM glucose stimulated Smad 2 mRNA expression that was 2.4-fold higher than those found in control cells cultured in 5.5 mM glucose at 24 h in NRK-52E cells. However, 29.5 mM mannitol did not change Smad 2 mRNA expression. High glucose-stimulated Smad 2 mRNA expression was abolished by the treatment of 10^{-6} M ANP.

In Figure 5, a high glucose concentration (35 mM) stimulated Smad 2/3 phosphorylation to level that was 350% higher than control cells cultured in 5.5 mM glucose in NRK-52E cells, whereas no significant change of Smad 2/3 phosphorylation was found in cells cultured in 29.5 mM D-mannitol. The treatment of 10^{-6} M ANP significantly reduced Smad 2/3 phosphorylation to the level that was 50% of high glucose-stimulated phosphorylation.

In Figure 6, immunocytochemistry demonstrated that phosphorylated Smad 2/3 nuclear translocation became markedly activated at 24 h after high glucose treatment. In contrast, cells cultured under normal glucose or D-mannitol treatment exhibited no increase in Smad2/3 nuclear translocation. Figure 6 also shows that high glucose-activated Smad2 nuclear translocation was blocked by the addition of 10^{-6} M ANP in cultured medium for 24 h.

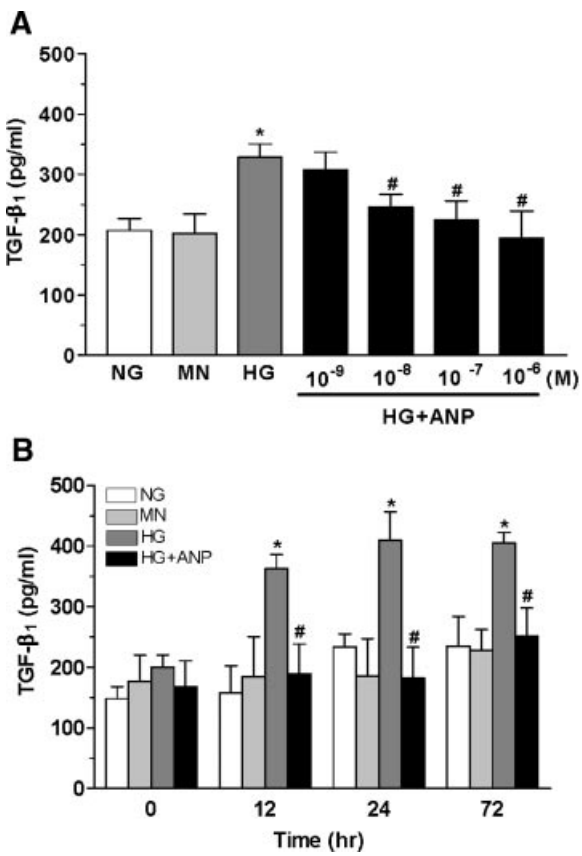

Fig. 3. ANP inhibited high glucose-stimulated the increase of TGF-β_1 protein measured by ELISA. **A**: Concentrations of TGF-β protein in the medium of NG (5.5 mM), HG (35 mM), MN (29.5 mM D-mannitol), and HG + ANP (ANP 10^{-8}–10^{-6} M) NRK-52E cells incubated for 24 h. **B**: Concentrations of TGF-β protein in the medium of NG (5.5 mM), HG (35 mM), MN (29.5 mM D-mannitol), and HG + ANP (ANP 10^{-6} M) NRK-52E cells incubated for 0, 12, 24, and 72 h. *$P < 0.05$ versus NG or MN; #$P < 0.05$ versus HG.

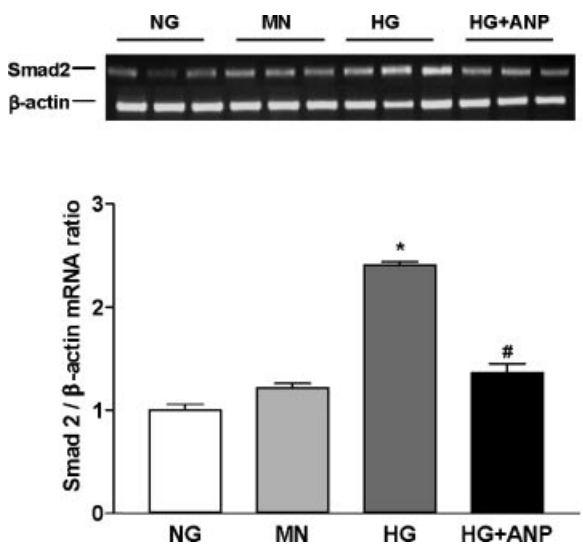

Fig. 4. ANP attenuated high glucose-stimulated Smad2 mRNA overexpression. Cells were incubated in normal glucose (NG, 5.5 mM), D-mannitol (MN, 29.5 mM), high glucose (HG, 35 mM), and high glucose plus ANP (HG + ANP 10^{-6} M) for 24 h. Cells were collected and Smad2 mRNA levels were assayed by RT-PCR. The relative densities of Smad2 were normalized with the β-actin level. Smad2 mRNA levels in NRK-52E incubated in normal glucose DMEM were considered as the control. Each point represents the mean ± SD of three independent experiments performed in triplicate. *$P < 0.05$ versus NG or MN; #$P < 0.05$ versus HG.

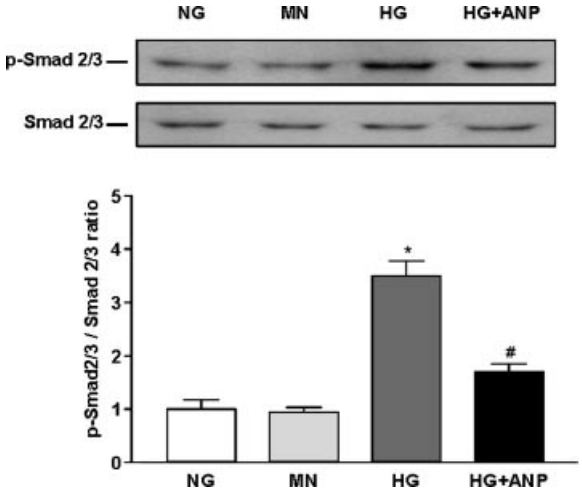

Fig. 5. ANP attenuated high glucose-stimulated the phosphorylation of Smad 2/3. Cells were incubated in normal glucose (NG, 5.5 mM), D-mannitol (MN, 29.5 mM), high glucose (HG, 35 mM), and high glucose plus ANP (HG + ANP 10^{-6} M) for 48 h. Cells were collected and assayed for Smad2/3 protein phosphorylation by SDS–PAGE and Western blot. The relative densities of phosphorylated Smad2/3 were normalized with the Smad2/3 control. Phosphorylated Smad2/3 protein levels in NRK-52E incubated in normal glucose DMEM were considered as the control. Each point represents the mean ± SD of three independent experiments. $^{*}P < 0.05$ versus NG or MN; $^{\#}P < 0.05$ versus HG.

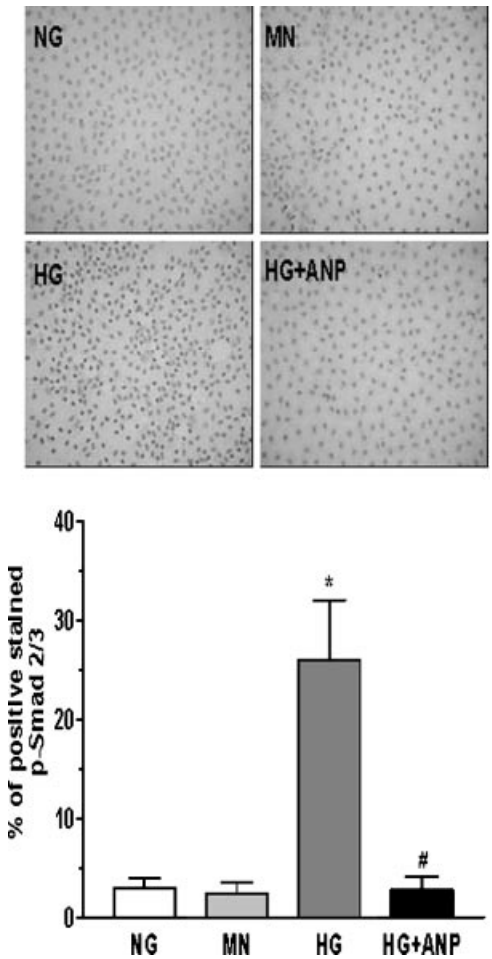

Fig. 6. ANP inhibited high glucose-enhanced phosphorylated Smad 2/3 (p-Smad 2/3) nuclear translocation. Cultured NRK-52E cells were treated in normal glucose (NG, 5.5 mM), D-mannitol (MN, 29.5 mM), high glucose (HG, 35 mM), and high glucose plus ANP (HG + ANP 10^{-6} M) for 24 h. **A**: Representative immunocytochemical photographs demonstrated p-Smad2/3 nuclear translocation (dark black nucleus) with p-Smad2/3 antibody. **B**: The percentage of positively stained cells with p-Smad2/3 antibody in the four groups. Magnification: 200×. $^{*}P < 0.05$ versus NG or MN; $^{\#}P < 0.05$ versus HG.

ANP Attenuated High Glucose-Stimulated Collagen Synthesis

Figure 7A shows that collagen type III mRNA expression was 2.5-fold increased in NRK-52E cell line cultured in 35 mM glucose at 24 h than that in cells cultured in 5.5 mM glucose, but no increase of collagen type III mRNA expression was found in the cells cultured with 29.5 mM mannitol. The up-regulation of collagen type III mRNA expression by high glucose was significantly reduced in a dose-dependent manner by the treatment of 10^{-8}–10^{-6} M ANP at 24 h. Figure 7B shows that the addition of 10^{-6} M ANP could significantly inhibit high glucose-stimulated collagen type III mRNA expression in a time-dependent manner. In Figure 8, 35 mM glucose significantly increased collagen type I mRNA expression at 24 h in NRK-52E cell line to the level that was 2.3-time higher as compared to cells cultured in 5.5 mM glucose, while no increase of collagen type I mRNA expression was found in the cells cultured with 29.5 mM mannitol. The increase of collagen type I mRNA expression by high glucose was significantly reduced by the addition of 10^{-6} M ANP at 24 h. In Figure 9, collagen type I protein level was 385% higher in NRK-52E cells cultured in 35 mM glucose than that in 5.5 mM glucose at 24 h, while 29.5 mM D-mannitol did not affect the collagen type I protein level. High glucose-stimulated collagen type I protein production was blocked by the addition of 10^{-6} M ANP.

Guanylate Cyclase Inhibitor Attenuated the Inhibition of ANP on High Glucose-Stimulated Synthesis of TGF-β_1

Figure 10 shows that the enhancement of TGF-β_1 mRNA by high glucose was significantly reduced by the treatment with 10^{-6} M ANP at 24 h. However, the inhibitory effect of ANP on high glucose-induced up-regulation of TGF-β_1

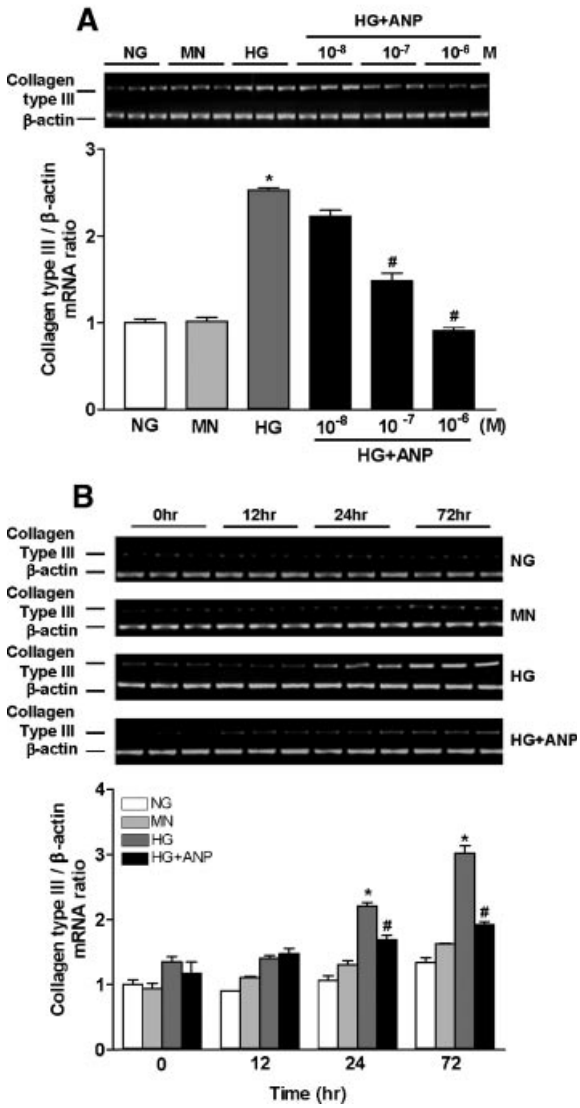

Fig. 7. ANP inhibited high glucose-stimulated expression of Collagen type III mRNA. **A**: Collagen type III mRNA levels are assayed by RT-PCR in NRK-52E cells which were incubated in DMEM with normal glucose (NG, 5.5 mM), high D-glucose (HG, 35 mM), D-mannitol (MN, 29.5 mM), or 35 mM D-glucose plus ANP (HG + ANP 10^{-8}–10^{-6} M) for 24 h. **B**: Collagen type III mRNA levels in NG, MN, HG, and HG + ANP (10^{-6} M) NRK-52E cells, incubated for 0, 12, 24, and 72 h. Cells were collected and assayed for rat collagen type III mRNA levels by RT-PCR. The relative densities of collagen type III were normalized with the β-actin control. Collagen type III mRNA levels in NRK-52E incubated in normal glucose DMEM were considered as the control. Each point represents the mean ± SD of three independent experiments performed in triplicate. $^{*}P < 0.05$ versus NG or MN; $^{\#}P < 0.05$ versus HG.

gene was attenuated when the guanylate cyclase inhibitor, LY-83583, was added into the medium. Furthermore, we measured the concentration of TGF-β_1 in cell culture media by ELISA. Figure 11 shows that guanylate cyclase

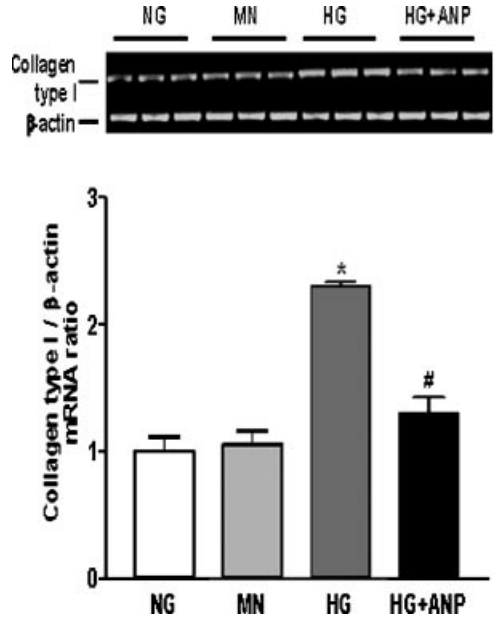

Fig. 8. ANP attenuated high glucose-stimulated expression of Collagen type I mRNA. Collagen type I mRNA levels are assayed by RT-PCR in NRK-52E cells which were incubated in DMEM with normal glucose (NG, 5.5 mM), high D-glucose (HG, 35 mM), D-mannitol (MN, 29.5 mM), or 35 mM D-glucose plus ANP (HG + ANP 10^{-6} M) for 24 h. The relative densities of collagen type I were normalized with the β-actin control. Collagen type I mRNA levels in NRK-52E incubated in normal glucose DMEM were considered as the control. Each point represents the mean ± SD of three independent experiments performed in triplicate. $^{*}P < 0.05$ versus NG or MN; $^{\#}P < 0.05$ versus HG.

inhibitor significantly blocked the inhibition effect of ANP on high glucose-stimulated overproduction of TGF-β_1 protein.

DISCUSSION

In the present study, we demonstrated that ANP can attenuate the increase of TGF-β1 synthesis, Smad2/3 phosphorylation and collagen synthesis in renal proximal epithelial cells by the stimulation of high glucose in vitro. In addition to its regulation of blood pressure and blood volume [de Bold et al., 1981; Brenner et al., 1990], several investigations have indicated that ANP may also exert a cytoprotective effect on several cell types, such as hepatocytes [Gerbes et al., 1998; Kiemer et al., 2000], cardiomyocytes [Kato et al., 2005; Nishikimi et al., 2006], endothelial cells [Kiemer et al., 2002, 2003; Irwin et al., 2005], vascular smooth muscular cells [Baldini et al., 2005], and macrophage [Tsukagoshi et al., 2001]. These functions mainly inhibit hypertrophy, and fibrosis which is caused from cell damage by hypoxia, ischemia-reperfusion, inflammation, and drugs

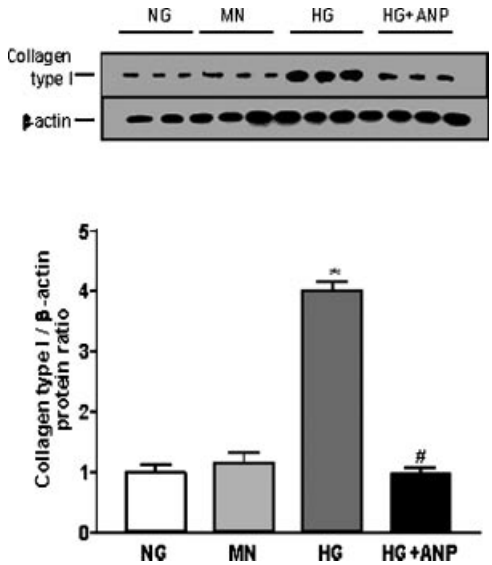

Fig. 9. ANP inhibited high glucose-stimulated production of collagen type I. Collagen type I protein levels are assayed by SDS–PAGE and Western blot in NRK-52E cells which were incubated in DMEM with normal glucose (NG, 5.5 mM), high D-glucose (HG, 35 mM), D-mannitol (MN, 29.5 mM), or 35 mM D-glucose plus ANP (HG + ANP 10^{-6} M) for 24 h. The relative densities of collagen type I protein were normalized with the β-actin control. Collagen type I protein levels in NRK-52E incubated in normal glucose DMEM were considered as the control. Each point represents the mean ± SD of three independent experiments performed in triplicate. $^{*}P < 0.05$ versus NG or MN; $^{\#}P < 0.05$ versus HG.

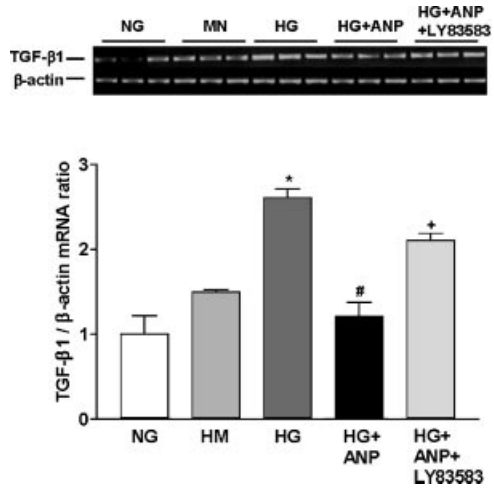

Fig. 10. Guanylate cyclase inhibitor reversed the inhibition of ANP on high glucose-stimulated expression of TGF-β_1 mRNA. TGF-β1 mRNA levels are assayed by RT-PCR in NRK-52E cells which were incubated in DMEM with normal glucose (NG, 5.5 mM), high D-glucose (HG, 35 mM), or D-mannitol (MN, 29.5 mM), 35 mM D-glucose plus ANP (HG + ANP 10^{-8}–10^{-6} M), and 35 mM D-glucose plus 10^{-6} M ANP and 10^{-7} M LY83583 for 24 h. The relative densities of TGF-β1 were normalized with the β-actin control. TGF-β1 mRNA levels in NRK-52E incubated in normal glucose DMEM were considered as the control. Each point represents the mean ± SD of three independent experiments performed in triplicate. $^{*}P < 0.05$ versus NG or MN; $^{\#}P < 0.05$ versus HG; $^{+}P < 0.05$ versus HG + ANP.

[Hannken et al., 2001; Polte et al., 2002]. TGF-β is an important growth factor involving hyperglycemia-induced vascular and renal complications in diabetes [Sharma and Ziyadeh, 1995]. In this study, ANP was confirmed to capably inhibit the increase of TGF-β1 and collagen synthesis in high glucose condition. This is the first to indicate that ANP might protect renal cells from hyperglycemic injury as it protects other cells from other detrimental mediators, such as hypoxia, ischemia, inflammation, and vasoconstrictors.

In this study, we found that high glucose markedly stimulated TGF-β1 synthesis in a concentration-dependent manner in cultured proximal tubular epithelial cells. Several in vitro and in vivo studies have further demonstrated that the activation of TGF-β-dependent Smad production is one intermediate pathway to mediate high glucose-stimulated ECM synthesis in renal cells and in diabetic mice [Nakao et al., 1997]. In experimental animal models with type 1 and 2 diabetes, TGF-β mRNA and protein levels are increased in glomerular and tubular compartments [Gilbert et al., 1998; Hill et al., 2000], even in renal biopsy specimens from patients with proven diabetic renal disease [Iwano et al., 1996]. By using specific neutralizing monoclonal antibodies for TGF-β as antagonist, high glucose-induced rise in matrix expression, and Smad2/3 phosphorylation was significantly abolished in cultured renal cells [Ziyadeh et al., 1994; Li et al., 2003] as well as in diabetic animals [Sharma et al., 1996]. In the current study, we also observed that high glucose stimulated Smad2/3 phosphorylation and collagen synthesis in renal proximal epithelial cells. Similar to TGF-β antibodies, the addition of ANP could also attenuate high glucose-activated smad2/3 phosphorylation, and collagen types I and III synthesis while the increase of TGF-β synthesis was also abolished by ANP.

In addition to being synthesized in the atrium, ANP has also been reported to be secreted by many other tissues including cardiac ventricles [Shin et al., 1997], kidneys [Lee et al., 1996; Shin et al., 1997, 1998], and

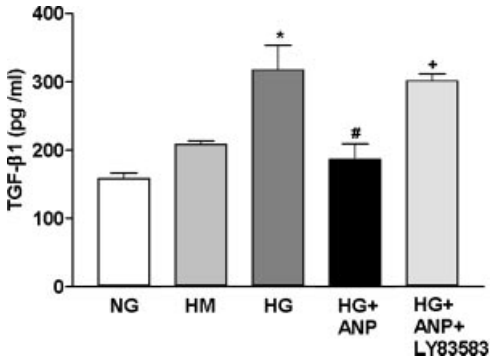

Fig. 11. Guanylate cyclase inhibitor reversed the inhibition of ANP on high glucose-stimulated expression of TGF-β_1 protein. TGF-β1 protein levels are assayed by ELISA in NRK-52E cells which were incubated in DMEM with normal glucose (NG, 5.5 mM), high D-glucose (HG, 35 mM), D-mannitol (MN, 29.5 mM), or 35 mM D-glucose plus ANP (HG + ANP 10^{-8}–10^{-6} M), and 35 mM D-glucose plus 10^{-6} M ANP and 10^{-7} M LY83583 for 24 h. Results represent means ± SD for four independent experiments from different cell preparations. $^{*}P < 0.05$ versus NG or MN; $^{\#}P < 0.05$ versus HG; $^{+}P < 0.05$ versus HG + ANP.

adrenal glands [Lai et al., 2000] in several disorders. In contrast to ANP production from cardiac atrium, ANP synthesis is markedly increased from extra-cardiac tissues. The increase of extra-atrial ANP was not recognized to be caused by volume expansion or increased stretch, but to result from local ischemia, or hypoxia. Certainly, the amount in local organs is possibly too low to induce endocrine effect. Therefore, ANP may act locally at the sites of its synthesis through autocrine and/or paracrine action [Nishikimi et al., 2006]. As described above, ANP synthesis increase in diabetic kidney [Shin et al., 1997; Lai et al., 2002], particularly, in proximal tubular cells, distal collect duct and medullary collect duct cells [Lai et al., 2002]. In addition to the increase of renal- and cardiac ventricle-derived ANP, plasma ANP concentration has also been found to increase in diabetic humans and rats [Bell et al., 1989; Shin et al., 1997]. Therefore, the alteration of circulating or local ANP in diabetic animals might possibly modulate high glucose-induced cell injury, including that found in high glucose-activated TGF-β1, collagen type I and III synthesis, and Smad2/3 phosphorylation in cultured proximal tubular epithelial cells in this study.

High glucose is the key initiator to the development of diabetic complications through several complex signal transduction pathways, such as mitogen-activated protein kinases (MAPK), NF-κB and angiotensin II. These high glucose-activated signal pathways then increase various growth factors, cytokines, and chemcytokines [Wolf, 2004]. In disorders with hypoxia, ischemia, inflammation and high sodium overload, exogenous addition of ANP has been found capable of down-regulating p38 MAPK [Tsukagoshi et al., 2001; Kiemer et al., 2003], NF-κB [Kiemer et al., 2000, 2002; Tsukagoshi et al., 2001; Irwin et al., 2005] and local angiotensin II production [Rosón et al., 2006]. Therefore, it is reasonable to hypothesize that the attenuation of high glucose-enhanced TGF-β1 synthesis, Smad2/3 phosphorylation and collagen synthesis after ANP treatment might also be mediated by the down-regulation of p38 MAPK, NF-κB and ANG II. Among these pathways, angiotensin II blockade has served as a mainstay therapy used to prevent the progression of diabetic nephropathy [Strippoli et al., 2006]. In rat kidney proximal tubular cells, TGF-β1 gene expression has been stimulated in high levels of glucose by the activation of angiotensinogen expression via p38 MAPK pathway [Zhang et al., 2000, 2002]. In the kidneys of Zucker Obese diabetic rats, TGF-β1 synthesis was also increased, and the increase could be abolished by angiotensin II converting enzyme inhibitors [Sharma et al., 2006]. ANP was found to capably attenuate ANG II-induced hypertrophy of renal tubular cells [Hannken et al., 2001] and also acute sodium overload-induced ANG II over-production in normal rat kidneys [Rosón et al., 2006]. Based on these findings, it was worthy investigating whether the attenuation of high glucose-enhanced TGF-β1 synthesis, Smad2/3 phosphorylation and collagen synthesis after ANP treatment is mediated by the inhibition of MAPK-activated local ANG II production via ANP-stimulated MAPK phosphatase-1 (MKP-1).

In the current study, the addition of guanylate cyclase inhibitor, LY83583, was able to abolish the attenuation effect of ANP on high glucose-induced activation of TGF-β synthesis in proximal renal cells. When ANP binds to natriuretic peptide receptors (NPR-A and NPR-B), it can activate particulate guanylate cyclase (pGC) and cGMP production is in turn increased. However, Akiho et al. [1994] reported that ANP could also relax vascular smooth muscle cells via soluble guanylase cyclase (sGC). Additionally, several investigators have demonstrated that both ANP-gGC-cGMP and

NO-sGC-cGMP systems have complementary roles to cooperatively regulate the important pathophysiological functions [Hussain et al., 2001; Madhani et al., 2006]. By this negative-feedback interaction between ANP and NO, an excess in one mediator could be compensated by the other to prevent overactivation of cGMP signaling in one cell type. In this study, the addition of exogenous ANP caused high ANP concentration in cultured medium to activate gGC-cGMP pathway. Accordingly, cGMP level in NRK-52E cells which was inhibited by the addition of LY83583 should result from exogenous ANP. Therefore, this present experiment implicates that the attenuation of high glucose-enhanced TGF-β1/Smads/collagen signaling is at least in part mediated by ANP-activated cGMP.

In summary, our study showed that exogenous ANP could significantly inhibit the activation of TGF-β1 synthesis, Smad2/3 phosphorylation and collagen synthesis in renal proximal epithelial cells under high glucose.

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