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Cyclic AMP inhibits stretch-induced overexpression of fibronectin in glomerular mesangial cells

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Abstract

Glomerular hypertension is proposed to play an important role in the progression of various glomerular diseases. Glomerular mesangial cells are considered to be exposed to the stretch stress due to glomerular hypertension and are found to produce the excess amount of extracellular matrix (ECM) proteins including fibronectin when exposed to the mechanical stretch. Herein, we provide the evidence that cAMP-generating agents inhibit the stretch-induced overexpression of fibronectin through the inhibition of the stretch-induced activation of mitogen-activated protein kinases (MAPKs) in protein kinase-A-dependent manner. We also found that the mechanical stretch enhanced the binding of nuclear extracts to activator protein-1 (AP-1)-like sequences in the promoter region of rat fibronectin gene and this enhancement was also prevented by the cAMP-generating agent. These results indicate that the agents, which activate cAMP/protein kinase-A axis, may work protectively against the injury from glomerular hypertension in mesangial cells.

Keywords: cAMP; Mechanical stretch; Glomeruloscrelosis; MAP (mitogen-activated protein) kinase; AP-1 (activator protein-1); Fibronectin

1. Introduction

Glomerular hypertension due to intrarenal hemodynamic alterations has been proposed to play an important role in the pathogenesis of not only an increase in the glomerular permeability to macromolecules (Osterby et al., 1990; Riser et al., 2000), but also an enhancement of the accumulation of extracellular matrix (ECM) proteins (Riser et al., 2001; Kawata et al., 1998). In the glomeruli, mesangial cells are considered to be a primary target for the insult induced by increased glomerular capillary pressure (Campeanu et al., 1973; Hostetter et al., 1982; Osterby et al., 1990) and play a crucial role in the glomerular trafficking of plasma proteins, their deposition, and extracellular matrix (ECM) protein accumulation within the mesangium, leading to the development and progression of glomerular sclerotic lesions in various glomerular diseases such as diabetic nephropathy (Fogo, 1999; Fornoni et al., 2000; Kawata et al., 1998). Recent studies suggest that the mechanical stretch to mesangial cells, an in vitro model for glomerular hypertension, induces the proliferation of mesangial cells and the production of ECM proteins (Cortes et al., 1997, 1999; Hirakata et al., 1997; Ishida et al., 1999; Yasuda et al., 1996).

The members of mitogen-activated protein kinases (MAPKs) superfamily, extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK) and p38 MAPK, play a key role in the signaling in response to various stimuli (Cobb, 1999; Garrington and Johnson, 1999; Schaeffer and Weber, 1999). MAPKs were found to be activated in mesangial cells by vasoactive peptides, cytokines and high glucose concentrations, (Araki et al., 1997; Guan et al., 1996, 1998; Haneda et al., 1997; Isono et al., 1998; Kang et al., 1999). Of the members of MAPK superfamily, we have reported that the mechanical stretch activates ERK and JNK and enhances the production of transforming growth factor-β1 (TGF-β1) and fibronectin in mesangial cells (Ishida et al., 1999). The activated ERK phosphorylates Elk-1, one of the ternary complexes, and induces the expression of c-fos, followed by the enhancement of DNA binding activity of activator protein-1 (AP-1) with c-jun phosphorylated by JNK, leading to the enhancement of the expression of a

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wide variety of genes including ECM proteins (Kreisberg et al., 1994; Rosenfeldt et al., 1998). Indeed, we have shown that an inhibition of ERK by a specific inhibitor of MAPK or ERK kinase (MEK) could prevent the mechanical stretch-induced expression of TGF-β1 and fibronectin by reducing the DNA binding activity of AP-1 (Ishida et al., 1999). In addition, the activation of p38 MAPK was also found in mesangial cells exposed to the mechanical stretch (Gruden et al., 2000; Ingram et al., 1999b). Thus, the activation of MAPKs is considered to play a central role in the signaling of the mechanical stretch in mesangial cells.

Recently, cGMP, provided by a nitric oxide (NO) donor, has been reported to inhibit the mechanical stretch-induced activation of MAPKs in mesangial cells through the disruption of the actin cytoskeleton (Ingram et al., 2000a). We and others have shown that the agonist-induced activation of MAPKs was inhibited by cAMP-generating agents as well as by cGMP-rising agents (Haneda et al., 1996; Ingram et al., 2000b). Therefore, we hypothesized that cAMP-generating agents could inhibit the mechanical stretch-induced activation of MAPKs and thus prevent the mechanical stretch-induced overproduction of ECM proteins. To test this hypothesis, we examined the effect of cAMP-generating agents, dibutyryl-cAMP and beraprost sodium, on the mechanical stretch-induced activation of MAPKs, AP-1 DNA binding activity, and fibronectin mRNA expression in cultured rat glomerular mesangial cells.

2. Materials and methods

2.1. Materials

Beraprost sodium was kindly donated by Yamanouchi Pharmaceutical (Tokyo, Japan), and dibutyryl-cAMP was purchased from Sigma (St. Louis, MO, USA). [α-^{32}P] dCTP and [γ-^{32}P] ATP were bought from New England Nuclear (Boston, MA, USA). Anti-active JNK antibody and consensus oligonucleotides of AP-1 were purchased from Promega (Madison, WI, USA). Anti-active ERK antibody and anti-active p38 MAPK antibody were bought from New England Bio-Lab (Beverly, MA, USA). Anti-ERK2, anti-JNK1and anti-p38 MAPK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). KT5720 was bought from BIOMOL (Plymouth Meeting, PA, USA).

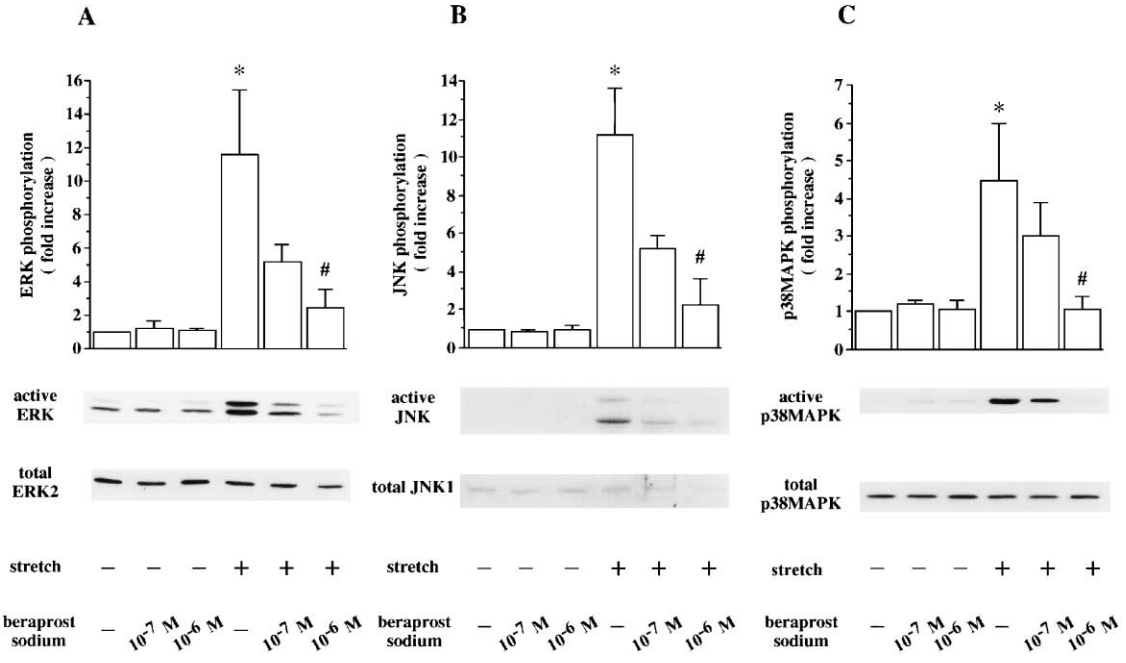


Fig. 1. Effect of beraprost sodium on stretch-induced phosphorylation of ERK, JNK and p38 MAPK in mesangial cells. Mesangial cells were incubated with various concentrations of beraprost sodium for 10 min and then exposed to the mechanical stretch 15 min (60 cycles/min, 20% elongation). (A) The degree of ERK activation quantified by a densitometric analysis is shown on the upper panel. A representative result of Western blot analysis for active ERK by anti-phospho ERK antibody and for total ERK by anti-ERK antibody is shown on the lower panel. Mean ± S.D. ($N=3$). * $P<0.05$ vs. nonstretched cell, # $P<0.05$ vs. stretched cell without beraprost sodium. (B) The degree of JNK activation quantified by a densitometric analysis is shown on the upper panel. A representative result of Western blot analysis for active JNK by anti-phospho JNK antibody and for total JNK by anti-JNK antibody is shown on the lower panel. Mean ± S.D. ($N=3$). * $P<0.05$ vs. nonstretched cell, # $P<0.05$ vs. stretched cell without beraprost sodium. (C) The degree of p38 MAPK activation quantified by a densitometric analysis is shown on the upper panel. A representative result of Western blot analysis for active p38 MAPK by anti-phospho p38 MAPK antibody and for total p38 MAPK by anti-p38 MAPK antibody is shown on the lower panel. Mean ± S.D. ($N=3$). * $P<0.05$ vs. nonstretched cell, # $P<0.05$ vs. stretched cell without beraprost sodium.

2.2. Cell culture and experimental protocol

Mesangial cells were obtained from a culture of glomeruli isolated from male Sprague–Dawley rats weighing 100–150 g. Mesangial cells were maintained in RPMI 1640 medium, supplemented with 20% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin and ITS (insulin, transferrin, selenious acid) premix at 37 °C in 5% CO_2 (Kikkawa et al., 1987). Mesangial cells from the 4th to 9th passages were plated on six-well plates with a flexible-bottom coated with type 1 collagen. Subconfluent cells were made quiescent by incubating in RPMI 1640 medium containing 0.2% bovine serum albumin for 24 h and subjected to the cyclic stretch. Stretch was mediated by controlled cycles of vacuum applied to the underside of the flexible-bottom culture well using a computer-assisted system (Flexercell Unit; Flexercell). In the present study, we provided cyclic stretch to mesangial cells at 20% elongation and 60 cycles/min for 15 min, since others and we found that this intensity induced maximal activation of MAPKs in mesangial cells (Hirakata et al., 1997; Ishida et al., 1999). Furthermore, mesangial cells were exposed to mechanical stretch for 1 h in measurement of DNA binding activity and for 12 h in measurement of the expression of fibronectin gene. In some experiments, cells were incubated with dibutyryl-cAMP or beraprost sodium for 10 min prior to the exposure to the stretch stress. KT5720, an inhibitor of protein kinase-A, was added 30 min prior to the treatment with beraprost sodium.

2.3. Western blot analysis

Mesangial cells were washed with cold phosphate-buffered saline (PBS) and harvested on ice using a solution containing 62.5 mM Tris–HCl (pH 6.8), 2% sodium lauryl sulfate (SDS), and 10% glycerol. The concentrations of the proteins were measured with the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA); 50 mM dithiothreitol and 0.1% bromphenol blue were added to the lysates. Equivalent amounts of proteins were separated by electrophoresis using a 10% SDS–polyacrylamide gel and transferred to polyvinylidene difluoride Immobilon-P membrane (Millipore, Bedford, MA, USA). After blocking with 5% bovine serum albumin and 0.1% Tween-20 in PBS at 4 °C overnight, membranes were incubated with anti-active ERK antibody (1:1000), anti-active p38 MAPK antibody (1:1000) and anti-active JNK antibody (1:5000) in a dilution buffer (PBS containing 5% bovine serum albumin and 0.1% Tween-20) at 4 °C overnight. The membranes were washed three times with PBS containing 0.1% Tween-20 and then incubated with horse-radish peroxidase-conjugated anti-goat

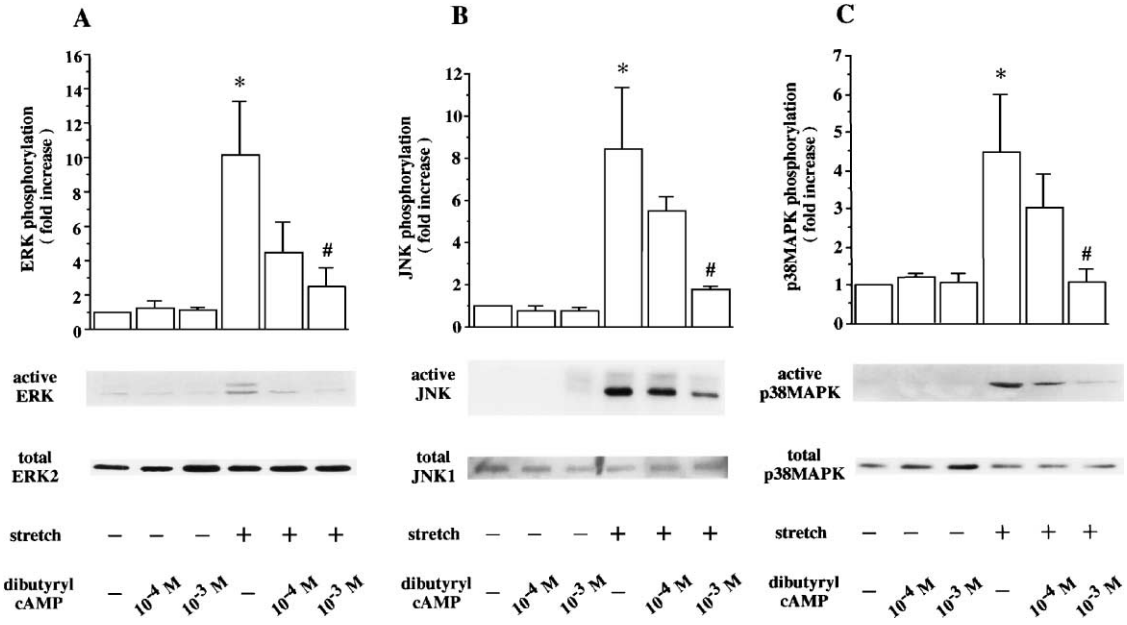


Fig. 2. Effect of dibutyryl-cAMP on stretch-induced phosphorylation of ERK, JNK and p38 MAPK in mesangial cells. Mesangial cells were incubated with various concentrations of dibutyryl-cAMP for 10 min and exposed to the mechanical stretch (60 cycles/min, 20% elongation) for 15 min. (A) The degree of ERK activation quantified by a densitometric analysis is shown on the upper panel. A representative result of Western blot analysis for active ERK by anti-phospho ERK antibody and for total ERK by anti-ERK antibody is shown on the lower panel. Mean ± S.D. ($N=3$). * $P<0.05$ vs. nonstretched cell, # $P<0.05$ vs. stretched cell without dibutyryl-cAMP. (B) The degree of JNK activation quantified by a densitometric analysis is shown on the upper panel. A representative result of Western blot analysis for active JNK by anti-phospho JNK antibody and for total JNK by anti-JNK antibody is shown on the lower panel. Mean ± S.D. ($N=3$). * $P<0.05$ vs. nonstretched cell, # $P<0.05$ vs. stretched cell without dibutyryl-cAMP. (C) The degree of p38 MAPK activation quantified by a densitometric analysis is shown on the upper panel. A representative result of Western blot analysis for active p38 MAPK by anti-phospho p38 MAPK antibody and for total p38 MAPK by anti-p38 MAPK antibody is shown on the lower panel. Mean ± S.D. ($N=3$). * $P<0.05$ vs. nonstretched cell, # $P<0.05$ vs. stretched cell without dibutyryl-cAMP.

immunoglobulin G (IgG)/anti-rabbit IgG at room temperature for 1 h. The bands were detected using enhanced chemiluminescence (Amersham, Buckinghamshire, UK). The membranes were reprobed with anti-ERK2, anti-p38 MAPK and anti-JNK1 antibody.

2.4. Northern blot analysis

Stretch-induced gene expression of fibronectin was examined by Northern blot analysis as previously described (Sugimoto et al., 1996). Total RNA isolated by a guanidium and phenol extraction (Trizol Reagent; Gibco, Grand Island, NY) was separated by formaldehyde/1.0% agarose gel electrophoresis and transferred onto a nylon membrane (Nytran; Schleider & Schuell, Dassel, Germany). After immobilizing the RNA by heating the membrane for 2 h at 80 °C and UV, the membrane was hybridized with rat fibronectin cDNA labeled with [α-^{32}P] dCTP (New England Nuclear) by a random primer method (BcaBEST; TAKARA, Sigma, Japan) in a buffer (0.5 M $NaPO_4$, pH 7.0, 1% bovine serum albumin, 7% SDS, and 1 mM EDTA) at 65 °C for 16 h. After the radioactive probes were stripped off the membrane, the membrane was rehybridized with radioactive probes of acidic ribosomal phosphoprotein PO (36B4) as an internal standard (Krowczynska et al., 1989).

2.5. Nuclear protein binding to AP-1 consensus and rat fibronectin/AP-1-like oligonucleotides

Nuclear extract was prepared as described by Sadowski and Gilmann (1993) with modifications. In brief, cells were lysed with hypotonic buffer (20 mM HEPES, pH 7.9, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstain) with 0.6% Nonidet P-40 and centrifuged at 16,000 × *g* for 20 min. Pellets were resuspended in high-salt buffer (hypotonic buffer with 420 mM NaCl and 20% glycerol), followed by rotating at 4 °C for 30 min and centrifuging at 16,000 × *g* for 20 min. The supernatants were used as nuclear proteins for a gel mobility shift assay. Nuclear proteins (3 μg) were incubated in a binding buffer [20 mM HEPES, pH 7.9, 1.8 mM $MgCl_2$, 2 mM dithiothreitol, 0.5 mM EDTA, 0.5 mg/ml bovine serum albumin and 2 μg poly (dI-dC)·poly (dI-dC)] at room temperature for

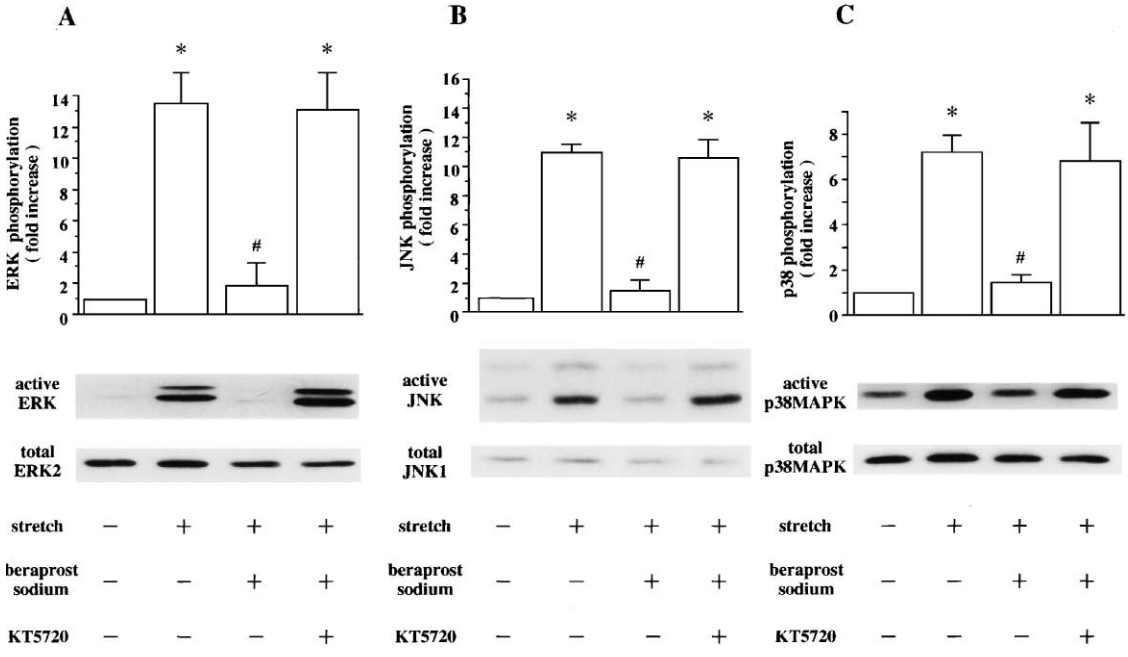


Fig. 3. Effect of KT5720 on mechanical stretch-induced MAPKs phosphorylation in mesangial cells. Mesangial cells were incubated with 10^{-6} M KT5720 for 30 min and with 10^{-6} M beraprost sodium for 10 min prior to the exposure to the mechanical stretch (60 cycles/min, 20% elongation) for 15 min. (A) The degree of ERK activation quantified by a densitometric analysis is shown on the upper panel. A representative result of Western blot analysis for active ERK by anti-phospho ERK antibody and for total ERK by anti-ERK antibody is shown on the lower panel. Mean ± S.D. ($N=3$). * $P<0.05$ vs. nonstretched cell and stretched cell with beraprost sodium, #$P<0.05$ vs. stretched cell without beraprost sodium. (B) The degree of JNK activation quantified by a densitometric analysis is shown on the upper panel. A representative result of Western blot analysis for active JNK by anti-phospho JNK antibody and for total JNK by anti-JNK antibody is shown on the lower panel. Mean ± S.D. ($N=3$). * $P<0.05$ vs. nonstretched cell and stretched cell with beraprost sodium, #$P<0.05$ vs. stretched cell without beraprost sodium. (C) The degree of p38 MAPK activation quantified by a densitometric analysis is shown on the upper panel. A representative result of Western blot analysis for active p38 MAPK by anti-phospho p38 MAPK antibody and for total p38 MAPK by anti-p38 MAPK antibody is shown on the lower panel. Mean ± S.D. ($N=3$). * $P<0.05$ vs. nonstretched cell and stretched cell with a beraprost sodium, #$P<0.05$ vs. stretched cell without beraprost sodium.

30 min, with or without unlabeled AP-1 consensus oligonucleotides or rat fibronectin/AP-1 oligonucleotides. The sequences of rat fibronectin/AP-1 oligonucleotides were 5′-TTCTCAGAGAGGTGACGCAATGTTCTCAA-3′ (positions −463 to −437 of the rat fibronectin promoter; the AP-1 motif is underlined). AP-1 consensus or rat fibronectin/AP-1 oligonucleotides end-labeled with [γ-^{32}P] ATP was added to the mixture at room temperature. The reaction mixture was then electrophoresed through 4% low anionic polyacrylamide gel and subjected to autoradiography. For the supershift experiments, nuclear protein extracts were preincubated with epitopes specific to c-jun and c-fos (Santa Cruz, CA, USA) for 30 min before the addition of the radioactive DNA probe.

2.6. Statistical analysis

Data are presented as mean values ± S.D. Analysis of variance (ANOVA) followed by Scheffe's test was used for multiple comparisons and $P<0.05$ level was considered significant.

3. Results

3.1. cAMP-generating agents inhibit the stretch-induced activation of MAPKs

First, we examined the effects of cAMP-generating agents on the stretch-induced activation of MAPKs. For this purpose, we used beraprost sodium, an analogue of prostaglandin I_2, which we previously found to increase intracellular cAMP, and dibutyryl-cAMP, a cell-permeable analogue of cAMP, in mesangial cells (Togawa et al., 1997; Haneda et al., 1996). The activities of MAPKs, ERK, JNK and p38 MAPK were all significantly enhanced in mesangial cells exposed to the stretch for 15 min (Figs. 1 and 2). Both beraprost sodium (Fig. 1) and dibutyryl-cAMP (Fig. 2) were able to inhibit the stretch-induced activation of MAPKs in a dose-dependent manner without affecting basal activities of MAPKs.

3.2. KT5720, a specific and cell-permeable inhibitor of protein kinase-A, abrogates the inhibitory effect of cAMP-generating agents on the stretch-induced MAPKs activity

To know the role of protein kinase-A in the inhibitory effect of dibutyryl-cAMP and beraprost sodium on the stretch-induced activation of MAPKs, we examined whether KT5720, a specific and a cell-permeable inhibitor of protein kinase-A, could attenuate the inhibitory effect of cAMP-generating agents. As shown in Fig. 3, the inhibitory effect of beraprost sodium on the stretch-induced activities of MAPKs (ERK, JNK and p38 MAPK) was significantly abrogated by treating mesangial cells with KT5720 for 30 min. Similarly, KT5720 for 30 min completely abrogated the inhibitory effect of dibutyryl-cAMP on the stretch-induced activation of MAPKs (data not shown).

3.3. Beraprost sodium inhibits stretch-induced expression of fibronectin mRNA

We and others found that the mechanical stretch could induce the expression of ECM proteins including fibronectin in mesangial cells (Ishida et al., 1999; Gruden et al., 2000) and this overexpression of ECM proteins is one of the major biological consequences of glomerular hypertension. Furthermore, we and the others have reported that the activation of MAPKs was responsible for the stretch-induced overexpression of fibronectin (Ishida et al., 1999; Ingram et al., 2000c; Gruden et al., 2000; Yasuda et al., 1996). Thus, to know the biological significance of the inhibitory effect of cAMP-generating agents on the stretch-induced activation of MAPK, we next examined the effect of beraprost sodium on the expression of fibronectin in mesangial cells exposed to the mechanical stretch. As shown in Fig. 4, mRNA expression of fibronectin was significantly enhanced in mesangial cells exposed to the mechanical stretch and this overexpression of fibronectin was almost completely inhibited by 10^{-6} M beraprost sodium. The inhibitory effect of beraprost sodium on the

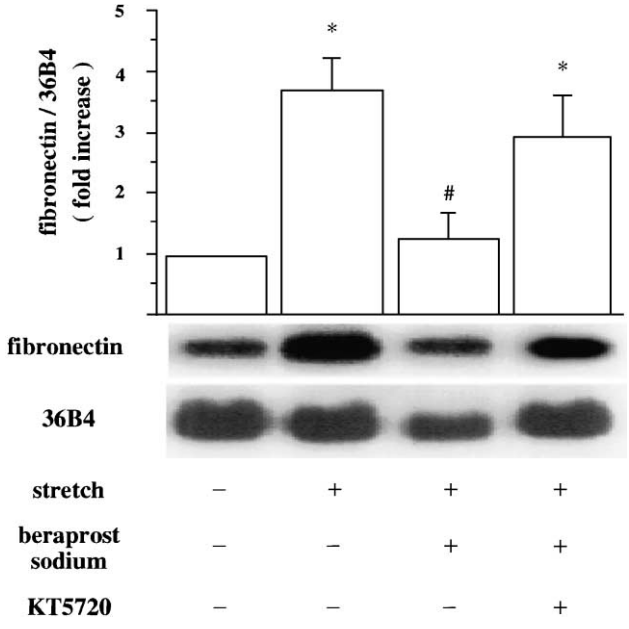
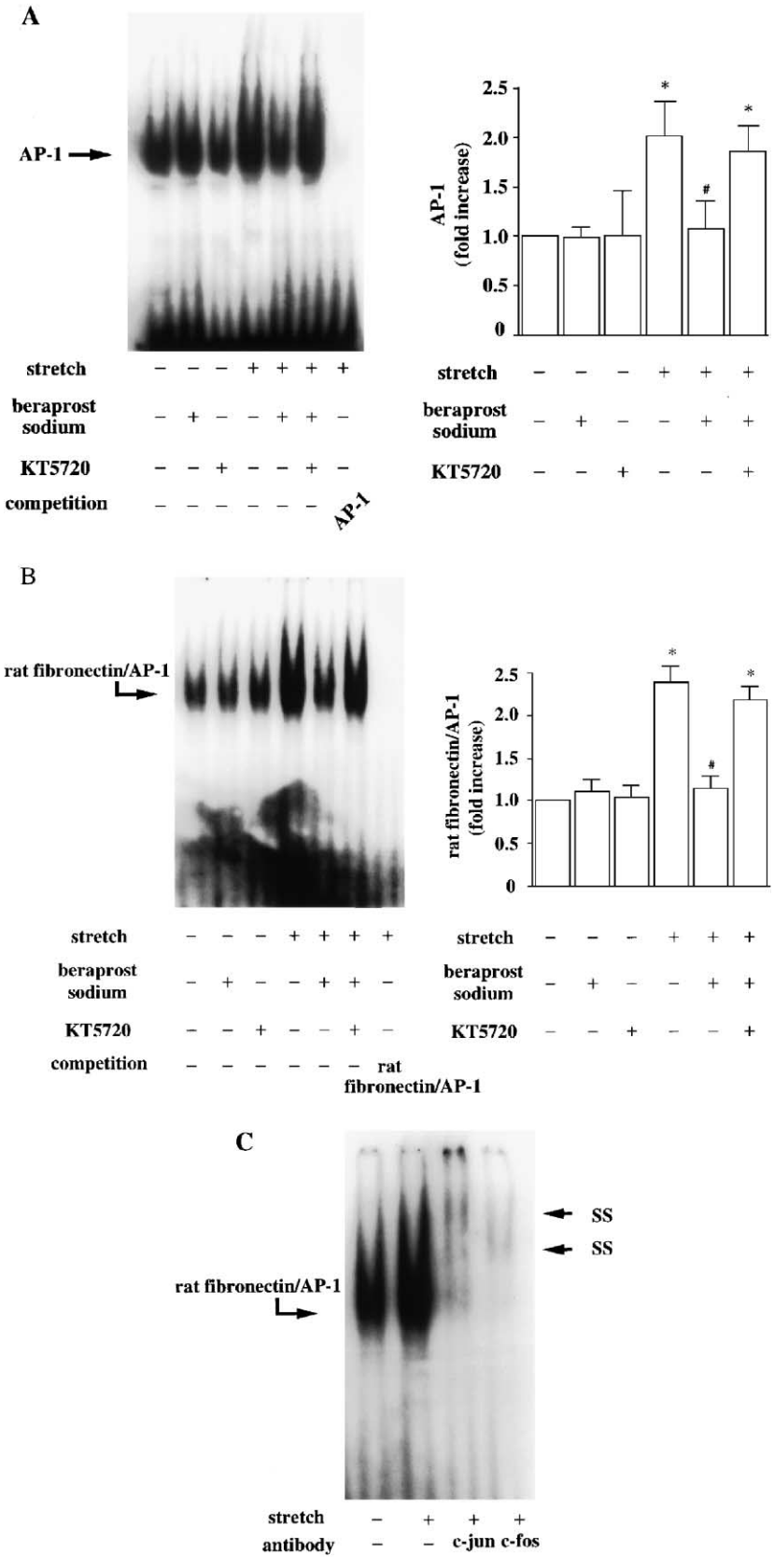


Fig. 4. Effect of beraprost sodium on stretch-induced expression of fibronectin mRNA in mesangial cells. Mesangial cells were treated with 10^{-6} M KT5720 for 30 min and with 10^{-6} M beraprost sodium for 10 min prior to the exposure to the mechanical stretch (60 cycles/min, 20% elongation) for 6 h. Total RNA (12 μg) was subjected to Northern blot analysis. The degree of fibronectin mRNA expression normalized by 36B4 mRNA expression quantified by a densitometric analysis is shown on the upper panel. A representative result of Northern blot for fibronectin and for 36B4 is shown on the lower panel. Mean ± S.D. ($N=4$). * $P<0.05$ vs. nonstretched cell and stretched cell with a beraprost sodium, # $P<0.05$ vs. stretched cell without a beraprost sodium.


A
AP-1
AP-1 (fold increase)
2.5
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#
stretch – – – + + + +
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C
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stretch – + + +
antibody – – c-jun c-fos

stretch-induced overexpression of fibronectin was again prevented by KT5720 (Fig. 4).

3.4. cAMP-generating agents inhibit the stretch-induced enhancement of nuclear protein binding activity to AP-1 and AP-1-like oligonucleotides

Because DNA binding activity of AP-1 was enhanced by the mechanical stretch through MAPKs-dependent manner (Ishida et al., 1999), we finally determined whether cAMP-generating agents could modulate DNA binding activity of AP-1. A representative result of a gel mobility shift assay is shown on the left panel of Fig. 5A and B, and we quantified the degree of DNA binding activity of AP-1 and rat fibronectin/AP-1 shown on the right panel of Fig. 5A and B. The mechanical stretch enhanced the binding of nuclear extracts to AP-1 consensus oligonucleotides, and this enhancement was inhibited by treating the cells with 10^{-6} M beraprost sodium (Fig. 5A). The inhibitory effect of beraprost sodium on the stretch-induced enhancement of AP-1 DNA binding activity was prevented by KT5720 (Fig. 5A). However, the promoter region of rat fibronectin gene does not contain consensus AP-1 binding site. Instead, AP-1-like binding site exists in the promoter region of rat fibronectin gene and this site was found to play a key role in fibronectin gene expression (Tamura et al., 1998). We thus carried out the gel mobility shift assay using rat fibronectin/AP-1-like site as the probe to confirm the role of stretch-induced nuclear protein binding activity in fibronectin expression. The nuclear extracts with ^{32}P-labeled rat fibronectin/AP-1-like site produced a single shifted band and the stretch increased the intensity of this band (Fig. 5B). The stretch-induced increase in nuclear binding activity to rat fibronectin/AP-1-like site was specifically competed by the unlabeled rat fibronectin/AP-1-like oligonucleotides (Fig. 5B). The stretch-induced increase in nuclear binding activity to rat fibronectin/AP-1-like site was inhibited by beraprost sodium and this inhibitory effect of beraprost sodium was prevented by KT5720. The protein–DNA binding complex formed by the rat fibronectin/AP-1-like site probe was supershifted by the antibody against c-fos or c-jun (Fig. 5C), indicating that c-fos and c-jun were the components of the protein–DNA complex formed in response to cyclic stretch.

4. Discussion

In the present study, we found that the mechanical stretch-induced activation of MAPKs (ERK, JNK and p38 MAPK) was inhibited by beraprost sodium, the cAMP-generating agent, and dibutyryl-cAMP, a cell-permeable analogue of cAMP, in rat glomerular mesangial cells. The mechanical stretch-induced increase in the expression of fibronectin was also inhibited by beraprost sodium. The mechanical stretch enhanced the nuclear protein binding to both consensus AP-1 site and AP-1-like site of the rat fibronectin promoter, and this increase in DNA binding activity was again inhibited by beraprost sodium. Furthermore, the inhibitory effect of beraprost sodium on the mechanical stretch-induced increase in the activities of MAPKs, AP-1 and rat fibronectin/AP-1 binding, and fibronectin mRNA expression was abrogated by KT5720, a specific and a cell-permeable inhibitor of cAMP-dependent protein kinase.

An increase in the intraglomerular pressure, glomerular hypertension, is proposed as one of the major factors responsible for the development and progression of glomerular injuries (Kawata et al., 1998). Glomerular mesangial cells are the crucial target cells exposed to the mechanical stretch caused by glomerular hypertension (Hostetter et al., 1982; Osterby et al., 1990). We and others have reported that the members of MAPKs superfamily, ERK, JNK and p38 MAPK, are activated in mesangial cells by the mechanical stretch (Ingram et al., 1999a,b,c; 2000c; Ishida et al., 1999). Since MAPKs are considered to play a central role in the signal transduction system induced by various stimuli, it is important to clarify the interaction between MAPKs and other signaling molecules such as cAMP and cGMP. In this study, we used beraprost sodium, an analogue of prostaglandin I_2, which we previously found to increase cellular cAMP in mesangial cells (Haneda et al., 1996). We clearly demonstrated that beraprost sodium and dibutyryl-cAMP could inhibit the mechanical stretch-induced activation of MAPKs through protein kinase-A-dependent manner.

There are several possible mechanisms by which cAMP inhibits the mechanical stretch-induced activation of MAPKs. First, cAMP may inhibit the activation of ERK by inhibiting Raf-1, since cAMP was found to inhibit ERK by preventing Ras-dependent activation of Raf-1 possibly

Fig. 5. Effect of beraprost sodium on stretch-induced activation of nuclear protein binding to AP-1 consensus or rat fibronectin/AP-1-like oligonucleotides in mesangial cells. Mesangial cells were treated with 10^{-6} M KT5720 for 30 min and with 10^{-6} M beraprost sodium for 10 min prior to the exposure to the mechanical stretch (60 cycles/min, 20% elongation) for 1 h. (A) Nuclear extracts were prepared from the cells and incubated with ^{32}P-end-labeled AP-1 consensus oligonucleotides probe and subjected 4% SDS PAGE. A representative result of a gel mobility shift assay is shown on the left panel. The arrow shows the specific binding of AP-1 consensus oligonucleotides. The degree of AP-1 DNA binding activity quantified by a densitometric analysis is shown on the right panel. Mean ± S.D. ($N=3$). * $P<0.05$ vs. nonstretched cell and stretched cell with a beraprost sodium, # $P<0.05$ vs. stretched cell without a beraprost sodium. (B) Nuclear extracts were prepared from the cells and incubated with ^{32}P-end-labeled rat fibronectin/AP-1-like oligonucleotides probe and subjected 4% SDS PAGE. A representative result of a gel mobility shift assay is shown on the left panel. The arrow shows the specific binding of rat fibronectin/AP-1-like oligonucleotides. The degree of rat fibronectin/AP-1 DNA binding activity quantified by a densitometric analysis is shown on the right panel. Mean ± S.D. ($N=3$). * $P<0.05$ vs. nonstretched cell and stretched cell with a beraprost sodium, # $P<0.05$ vs. stretched cell without a beraprost sodium. (C) Nuclear extracts were prepared from the cells and incubated with ^{32}P-end-labeled rat fibronectin/AP-1-like oligonucleotides probe and subjected 4% SDS PAGE. The arrow shows the specific binding of rat fibronectin/AP-1-like oligonucleotides. Analysis of an involvement of c-fos and c-jun in stretch-induced nuclear factor binding to the rat fibronectin/AP-1 element by supershift assay. In supershift assay, the arrowhead indicates the supershifted band.

through the phosphorylation of Raf-1 (Ciullo et al., 2001; Cook and McCormick, 1993; Hafner et al., 1994; Ramstad et al., 2000). The recent report suggests that the activation of Rap-1 by cAMP induces the association of Rap-1 with Raf-1 and inhibits Ras-dependent activation ERK (Schmitt and Stork, 2001). Another possibility is that protein kinase-A phosphorylates and inactivates RhoA, thereby disrupting the actin cytoskelton, similarly to the inhibition of MAPKs by cGMP (Ingram et al., 2000a). Indeed, cAMP was shown to induce actin stress fiber disassembly, myosin light chain dephosphorylation, loss of adhesion to the substratum, and cell shape change (Kreisberg and Venkatachalam, 1985; Kreisberg et al., 1997). Finally, we found that beraprost sodium could induce the expression of MKP-1 (Togawa et al., 1997; Haneda et al., 1999), a well-known attenuator of ERK activity by the dephosphorylation. Since MKP-1 was shown to dephosphorylate and inactivate both ERK and JNK (Bondello et al., 1999; Franklin and Kraft, 1997; Sugimoto et al., 1996), it seems likely that the induction of MKP-1 through cAMP/protein kinase-A axis might be responsible for the inhibitory effect of cAMP on the mechanical stretch-induced activation of ERK and JNK. Regarding p38 MAPK, the mechanical stretch-induced activation of p38 MAPK was shown to be protein kinase C-dependent (Gruden et al., 2000). Since cAMP was found to inhibit various actions of PKC (Ochi et al., 1995; Tamir and Isakov, 1991), this might be one of the mechanisms of the inhibition of p38 MAPK.

Fibronectin is an important component of the ECM proteins and is implicated functionally in the regulation of several cellular processes, including cell adhesion, migration, transformation, and growth, and pathologically in the progressive accumulation of ECM proteins in the glomerular mesangium (Mosher, 1989; Hynes, 1990). In the experimental animal models of diabetic nephropathy, the expression of fibronectin mRNA and protein has been shown to be increased in isolated glomeruli (Di et al., 1993; Grant et al., 1998). We and others have already reported that the expression of fibronectin gene was enhanced in mesangial cells exposed to the cyclic stretch, an in vitro model of glomerular hypertension seen in diabetic state (Ingram et al., 2000b; Ishida et al., 1999). Our findings provide the evidence for the first time that stretch-induced fibronectin expression was completely inhibited by cAMP/protein kinase-A axis, suggesting a beneficial effect of the cAMP-generating agents in the prevention of the process to glomeruloscrelosis. The studies on the other types of cells, showing that fibronectin expression was suppressed by elevating intracellular cAMP, support our findings (Burton et al., 1988; Bernath et al., 1990; Coats and Brecher, 1993).

AP-1 transcription factor complex has been implicated as a key factor in the signal transduction in response to a variety of hormones, growth factors and mechanical stretch, since AP-1 functions by binding to specific sequence elements within the promoter region of a variety of genes (Karin, 1996; Karin et al., 1997; Hill and Treisman, 1995). Recent reports indicate that three MAPKs phosphorylate transcription factors inducing c-fos and c-jun transcription (Janknecht and Hunter, 1997; Whitmarsh et al., 1997). c-fos and c-jun combine to form the AP-1 transcription complex that binds to and activates certain genes. Indeed, we found that the mechanical stretch enhanced AP-1 DNA binding in the present study and that this increased DNA binding was inhibited by the treatment with beraprost sodium, similarly to its inhibition of fibronectin gene expression. However, no consensus AP-1 bind site exists in the rat fibronectin promoter. Thus, to further know how the mechanical stretch-induced MAPKs activation induces AP-1 binding, we performed a gel mobility shift assay using AP-1-like site located on -463 to -437 of rat fibronectin promoter as a probe. Interestingly, we found for the first time that the mechanical stretch increased the binding of nuclear proteins to this rat fibronectin/AP-1-like site. Furthermore, the results of the supershift assay revealed that the rat fibronectin/AP-1 element-binding factor contained c-fos and c-jun, which could be formed through the MAPK signaling induced by the mechanical stretch. We also found that the activation of the DNA binding protein of rat fibronectin/AP-1-like site was inhibited by beraprost sodium and this inhibitory effect of beraprost sodium was abrogated by KT5720. Thus, it seems likely that the mechanical stretch-induced activation of MAPKs followed by an increase in the binding of nuclear proteins to AP-1-like site was suppressed by the activation of cAMP/protein kinase-A axis in response to beraprost sodium.

In conclusion, we have demonstrated that beraprost sodium inhibits the mechanical stretch-induced fibronectin expression as well as MAPKs-AP1 pathway by the activation of cAMP/protein kinase-A axis. Based on our findings, we can propose that beraprost sodium could be a protective agent, acting through cAMP/protein kinase-A activation, against glomerular hypertension, which is responsible for the development and progression of glomerulosclerosis in various glomerular diseases such as diabetes mellitus.

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