Adrenomedullin Impairs the Profibrotic Effects of Transforming Growth Factor- β 1 through Recruiting Smad6 Protein in Human Renal Tubular Cells

Haichang Huang^a, Chunyan Ma^a, Min Yang^a, Chaoshu Tang^b, Haiyan Wang^a

^aDivision of Nephrology, Peking University First Hospital & Institute of Nephrology, ^bInstitute of Cardiovascular Research, Peking University

Key Words

Adrenomedullin • Transforming growth factor- β • Fibrosis • Kidney • Smad

mediated renal tubulointerstitial ECM turnover via an antagonistic mechanism of inhibitory Smad in TGF- β ₁-elicited signaling.

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Abstract

Adrenomedullin (AM) was originally identified as a vasodilator peptide, and has recently been shown to be an antiproliferative factor in renal mesangial cells, suggesting that adrenomedullin may impair the progression of glomerulosclerosis. This study was to investigate the effect of adrenomedullin on transforming growth factor- β ₁ (TGF- β ₁)-stimulated cell growth, synthesis of extracellular matrix (ECM) components and the related molecular mechanism in a human tubular epithelial cell line HK-2. TGF- β ₁ inhibited cell proliferation induced by fetal bovine serum, but neither AM itself affected cell proliferation, nor did AM influence TGF- β ₁-caused cell growth arrest. However, AM beginning at 10⁻⁸ M alleviated the action of TGF- β ₁-stimulated cellular collagen synthesis and secretion of fibronectin into cell culture supernatant. Activation of Smad proteins is known to be the key signaling pathway of the profibrotic effect of TGF- β ₁. AM at 10⁻⁸ M exerted no effect on TGF- β ₁-induced Smad2 phosphorylation, but prevented the suppression of the inhibitory Smad6 protein by TGF- β ₁ and restored Smad2-Smad6 complex formation. Our results suggest that AM can attenuate TGF- β ₁-

Introduction

Adrenomedullin (AM), a vasodilator peptide that is ubiquitously expressed in various tissues, especially in cardiovascular tissues and in the kidney, lung and endocrine glands. It has multifunctional biological properties, of which its effect on the control of circulation and the natriuretic and diuretic properties seem to be the most predominant characteristics [1, 2]. There is increasing evidences that AM may have an important pathophysiological role in kidney diseases, because elevated plasma AM concentration was observed in patients with chronic renal failure [3], glomerulonephritis [4], IgA nephropathy [5], and diabetic nephropathy [6]. AM is suggested to be involved in maintaining cardiovascular and renal homeostasis as well as modulating the progression of chronic renal diseases [7]. Renal fibrosis is characterized by increased synthesis and

impaired degradation of extracellular matrix proteins (ECM) in glomerulus and tubulointerstitium, regulated by a variety of profibrotic factors. Thus far, the role and relevant mechanism of AM in the progression of chronic renal fibrosis remains unknown. Vasoconstrictor peptides such as angiotensin II (Ang II) and endothelin-1 (ET-1) promote glomerulosclerosis and renal interstitial fibrosis, while vasodilators such as prostaglandin E₂ (PGE₂) delay the progression of tissue fibrosis [8]. This led us to hypothesize that AM may attenuate renal fibrosis. Transforming growth factor- β (TGF- β) is a potent fibrogenic factor in the pathogenesis of renal fibrosis, and has been shown to regulate extracellular matrix turnover within the kidney in both experimental animal models and human glomerulonephritis [9, 10], by stimulation of the synthesis of ECM components such as fibronectin, laminin and collagens and/or inhibition of ECM protein degradation. Many studies have demonstrated that SMAD proteins are critical components of the signaling pathway regulating the transcription of target ECM genes by TGF β [11]. The present study was designed to investigate the effects of AM on the profibrotic action of TGF- β ₁ on human tubular epithelial cells and the related intracellular molecular mechanisms.

Materials and Methods

Cell culture

The present studies were performed with an immortalized human renal proximal tubular cell line HK2 (ATCC, USA, Cat#: CRL-2190) kindly provided by Professor RA Zager [12], (University of Washington, Seattle, WA, USA). These cells have been extensively characterized by various investigators and express many features of proximal tubular cells [13]. The cells were maintained in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT, USA), 100 IU/mL penicillin, and 100 μ g/L streptomycin. Cell cultures were kept in an incubator at 37°C with 5% CO₂. In all experiments, cells were serum-starved by incubation in RPMI 1640 medium containing 1% FBS for 24 hours before treatment with TGF- β ₁ (R&D Systems, Minneapolis, MN, USA) or AM (Phoenix Pharmaceuticals Inc. Phoenix, AZ, USA). Cells were pre-treated with AM 30 minutes prior to adding TGF- β ₁.

Cell proliferation assay

Cell growth was studied by [³H]-thymidine incorporation and total viable cell counting in separate assay. For the thymidine incorporation, 10³ cells per well of HK-2 cells were cultured in 96-well culture plates (Nunc, Naperville, IL, USA) and made quiescence as described above. Cell proliferation was induced by incubation with 10% FBS. Before the end of

the incubation period, the cells were pulse-labeled with 0.5 μ Ci per well of [³H]-thymidine (1 mCi/ml, Amersham-Pharmacia Asia, Hong Kong) for the last two hours. Cells were fixed in 5% trichloroacetic acid for 30 minutes, followed by sequential washing with trichloroacetic acid (three times) and with PBS (three times). Acid insoluble material was dissolved in 0.5 mol/L NaOH with 1% Triton X-100 and counted in a liquid scintillation counter. This experiment was repeated three times independently with six wells for each measurement.

In the cell counting assay, 2x10⁴ cells per well of HK-2 cells were plated into 24-well culture plates (Nunc, Naperville, IL, USA) and cultured as described above. After various treatments, cells were trypsinized and total viable cell number was determined using a hemocytometer. Cells from three wells were combined for an average number in each measurement, and the assay was repeated three times independently.

Collagen synthesis and collagen secretion

It has been demonstrated that ³H-proline is preferentially incorporated into newly synthesized collagens, and that collagen is degraded into a high-molecular polypeptide containing hydroxyproline by pepsin at 4°C, while other noncollagen proteins are completely degraded [14]. Therefore, total collagen synthesis and secretion is reflected by ³H-proline incorporation in cell extracts and the radioactivity of ³H-hydroxyproline in the culture medium. In this study, the method from Koyano [15] was slightly modified. Briefly, 10⁴ cells were seeded in each well of a 24-well plate (Nunc, Naperville, IL, USA). During the last 12-hour incubation period, 1 μ Ci ³H-proline (1 mCi/mL, Amersham-Pharmacia Asia, Hong Kong) was added to each well. At the end of the stimulation, the culture medium was removed; cells were digested for two hours by 1 mg/mL pepsin at 4°C and finally precipitated in 30% trichloroacetic acid. The precipitated cellular pellets were washed twice with ice-cold PBS and then precipitated in 10% trichloroacetic acid. The same procedure was used to precipitate the culture medium. The precipitates were suspended with 500 μ l of 0.5 N NaOH, 0.1% Triton X-100. Then the radioactivity was measured by liquid scintillation counting. These experiments were repeated independently three times.

ELISA for fibronectin secretion

Cells were plated into 35 mm dishes (Nunc, Naperville, IL, USA) at a concentration of 10⁴ per dish, and incubated in 1% FBS RPMI 1640 medium for 24 h for serum starvation before treatment. At the end of treatment, 50 μ l of culture medium supernatants were transferred into ELISA plates pre-coated with standard FN antigen (Sigma, St. Louis, MO, USA). A rabbit polyclonal anti-FN antibody (1:10,000 dilution, Cat# F3648, Sigma, St. Louis, MO, USA) was added and incubated for one hour at room temperature. After washing with PBS for three times, ELISA plates were incubated with a peroxidase-labeled goat anti-rabbit antibody (1:20,000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 minutes. The color reaction was developed with o-phenylenediamine and hydrogen peroxide for five minutes, stopped with 200 μ l of 2M H₂SO₄, and the absorption was measured at 450 nm on a microplate

reader. The amount of FN was determined from standard curves assayed simultaneously with the samples. These experiments were repeated three times independently with triplicate measurements for each supernatant.

Western blot analysis and immunoprecipitation

10⁶ cells were seeded into 100 mm dishes (Nunc, Naperville, IL, USA) and grown for 24 hours and then growth-arrested with 1% FBS RPMI 1640 medium for 24 hrs. After treatment, the cells were washed three times with ice-cold PBS and harvested in 500 μ l lysis buffer [50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS)] containing proteinase inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μ M leupeptin, 20 μ M pepstatin, and 10 μ M aprotinin]. The cell lysates were centrifuged at 12,000g for 10 minutes at 4°C, and the supernatant was collected. The protein content was determined by the Bradford protein assay. Samples were boiled with Laemmli sample buffer for five minutes, separated by 10% SDS-PAGE gel, and transferred onto Immobilon-P (polyvinylidene difluoride, PVDF) membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS)–Tween (0.1%) for two hours at room temperature, followed by incubation with the different primary antibodies, goat polyclonal anti-Smad2, mouse monoclonal anti-Smad4, goat polyclonal Smad7 (1:750; Santa Cruz, CA, USA), rabbit polyclonal anti-Smad6 (1:750, Zymed Lab, San Francisco, CA, USA) and mouse monoclonal anti-phospho-Smad2 (1:2000, Cell Signaling Technology, Beverly, MA, USA) overnight at 4°C, respectively. The membranes were washed three times in TBS-Tween-20 for five minutes, followed by incubation with the secondary antibody conjugated with HRP (Santa Cruz) for one hour at room temperature. The luminescence detection was performed with the ECL system according to the manufacturer's protocol (Amersham-Pharmacia Asia, Hong Kong). Quantitation of the bands of the autoradiogram was performed using densitometry analysis. These experiments were repeated independently three times.

For immunoprecipitation, 500 μ g protein of the cell lysate was incubated with 1 μ g anti-Smad2 antibody for at least 6 hours at 4°C in a rotation rocker. Then 100 μ l of Protein G-Agarose (Oncogene, Boston, MA, USA) was added to each tube for 6 hours to pull down the immunocomplexes. The immunocomplex pellet was washed three times with lysis buffer, then the pellets were suspended in 30 μ l Laemmli sample buffer and boiled for 5 min, separated by 10% SDS-PAGE, and blotted with anti-Smad4, anti-Smad6, anti-smad7, and anti-Smad2 antibody respectively.

Statistical analysis

Data are presented as the mean $\pm$ S.D. Statistical differences between experimental groups were determined by analysis of variance using the SSPS 11.0 for Windows (SPSS Inc.). A *P* value <0.05 was considered statistically significant.

Results

Effects of AM on TGF- β_1 -mediated cell growth in the HK-2 cell line

At first, we investigated if AM affected HK-2 cell proliferation or altered the TGF- β_1 -induced growth inhibition measured by ³H-thymidine incorporation and cell number. As shown in Figure 1, 10⁻⁸ M AM did not stimulate quiescent HK-2 cell proliferation (*P*>0.05), neither did it affect the proliferation stimulated by 10% FBS for 48 hr (*P*>0.05). However, 5 ng/mL TGF- β_1 strongly suppressed the cell proliferation stimulated by 10% FBS for 48 hrs (*P*<0.01), while the presence of various concentrations of AM (10⁻⁹ to 10⁻⁷M) had no influence on the TGF- β_1 -mediated inhibition of cell growth (*P*>0.05).

Effect of AM on the synthesis and secretion of extracellular matrix components stimulated by TGF- β_1

In order to address the question whether AM had any influence on profibrotic effects of TGF- β_1 , collagen synthesis and fibronectin (FN) secretions were determined in the presence or absence of AM. In comparison with the control, 5 ng/mL TGF- β_1 stimulated collagen synthesis and secretion in HK-2 cells for 48 hrs by 234% and 196% (*P*<0.01), respectively. AM at 10⁻⁹ M did not affect TGF- β_1 -stimulated collagen synthesis and secretion, while at 10⁻⁸ M AM, a reduction of TGF- β_1 induced collagen secretion by 30% was observed (*P*<0.05). When the concentration of AM was increased to 10⁻⁷ M, it inhibited both collagen synthesis and secretion by 57% (*P*<0.05) and 64% (*P*<0.01), respectively. But AM itself had no effect on collagen synthesis and secretion in HK-2 cells (Figure 2).

Next, we examined the effect of AM on TGF- β_1 -induced FN secretion. TGF- β_1 (5 ng/mL) stimulated FN secretion by twofold compared with the control, pre-incubation of the cells with 10⁻⁸ M AM, inhibited FN secretion from HK2 cells stimulated by TGF- β_1 for 48 hrs (*P*<0.05) whereas 10⁻⁸ M AM itself did not influence basal FN secretion (Figure 3).

AM did not affect TGF- β_1 -triggered Smad2 phosphorylation

In order to elucidate how AM inhibited TGF- β_1 -induced collagen synthesis and FN secretion, the signaling

Fig. 1. Effect of AM on cell growth inhibited by TGF- β_1 in HK-2 cell. Proliferation was determined by ^3H -thymidine incorporation (A) and cell numbers (B) as described in Materials and Methods. Data are presented as means $\pm$ S.D. of three independent experiments (* P <0.01 compared with control, ** P <0.01 compared with FBS).

Fig. 2. Effect of AM on collagen synthesis and secretion stimulated by TGF- β_1 . Collagen synthesis was reflected by ^3H -proline incorporation into cellular protein (open box), and secretion by the radioactivity of ^3H -hydroxyproline in the culture medium (filled box). The radioactivity of ^3H -proline and ^3H -hydroxyproline is expressed as cpm/10⁴ cells. These experiments were repeated independently three times (* P <0.01 compared with control, ** P <0.05, *** P <0.01 compared with TGF- β_1).

Fig. 3. Effect of AM on fibronectin secretion stimulated by TGF- β_1 . The concentration of FN was determined by ELISA. Experiments were repeated three times independently with triplicate measurements for each supernatant (* $P < 0.05$ compared with control, ** $P < 0.05$ compared with TGF- β_1).

Fig. 4. Effect of AM on Smad2 phosphorylation stimulated by TGF- β_1 . (A) Time course of Smad2 phosphorylation after TGF- β_1 incubation in HK-2 cells. 50 μ g of cell lysate protein were loaded and probed with antibodies against phospho-Smad2 and total Smad-2, respectively. (B) The cells were pre-incubated with 10^{-8} M AM for 30 minutes and then stimulated with TGF- β_1 for 60 minutes. Smad2 phosphorylation was detected by Western blot analysis. The lower panels in (A) and (B) show total Smad2 protein level.

AM abolished TGF β_1 -mediated decrease in Smad6 expression and Smad2/Smad6 complex

One potential pathway for AM inhibition of Smad signaling could be mediated by inducing the expression of inhibitory Smads (I-Smads) including Smad6 and Smad7. Therefore, we examined the effect of AM on the expression of I-Smads and the association of Smad2 with Smad6 or Smad7 in HK2 cells. TGF- β_1 down-regulated Smad6 expression within 1 hour. The expression of Smad6 remained suppressed at 4 hours and 8 hours, and then gradually returned to basal values by 24 hours (Fig.5A). Therefore, we tested the effect of AM on the TGF β_1 -mediated down-regulation of Smad6 at 4 hours.

pathway of TGF- β_1 was analyzed. It is well documented that TGF- β_1 , on binding to its receptors, initiates phosphorylation of receptor-activated Smad2 and/or Smad3. Thus, we examined the phosphorylation and activation of Smad2 by TGF- β_1 in HK2 cells by Western blot using a phospho-specific Smad2 antibody. At first, the kinetics of Smad2 activation after incubation with TGF- β_1 in HK2 cells was assessed. Smad2 phosphorylation was seen as early as 15 minutes, reached the peak at 60 minutes, and remained elevated for 4 hours after addition of TGF- β_1 (Figure 4A). To investigate whether AM interfered with Smad2 phosphorylation, HK2 cells were pretreated with AM for 30 minutes and then incubated with TGF- β_1 for 60mins. 10^{-8} M AM did not modulate TGF- β_1 -triggered Smad2 phosphorylation or total Smad2 expression in HK2 cells (Figure 4B).

TGF β_1 induced a remarkably decrease in Smad6 at 4 hours ($p < 0.01$), whereas pre-incubation with 10^{-8} M AM prevented TGF β_1 -mediated suppression of Smad6 protein ($p < 0.05$) (Fig.5B). We further assayed Smad2/Smad6 complex formation by immunoprecipitation. In accordance with the Smad6 protein level, 10^{-8} M AM abrogated TGF β_1 -mediated repression of Smad2/Smad6 complex formation at 4 hours ($p < 0.05$) (Fig. 6). There was no change in the association of Smad2 with Smad4 (data not shown). Smad7 protein was undetectable in HK-2 cells at basal conditions or after various treatments with TGF β_1 .

Fig. 5. Effect of AM on the expression of Smad6 protein stimulated by TGF- β_1 . (A) Changes of Smad6 protein after TGF- β_1 stimulation for different time periods. 50 μ g protein from cell lysates were used. (B) Cells were pre-incubated with 10^{-8} M AM and then stimulated by TGF- β_1 for 4 hours (# $P<0.01$ compared with control, * $P<0.05$ compared with TGF- β_1). The lower panels in (A) and (B) show GAPDH protein as internal control.

Fig. 6. Effect of 10^{-8} M AM on the formation of Smad2-Smad6 complexes stimulated by TGF- β_1 . Cells were pre-incubated with 10^{-8} M AM and then stimulated by TGF- β_1 for 4 hours. The upper panel: For detection of Smad2-Smad6 complex, 500 μ g protein of the cell lysates were immunoprecipitated with or without anti-Smad2 antibody and then subjected to Western blot analysis probed with anti-Smad6 antibody (# $P<0.01$ compared with control, * $P<0.05$ compared with TGF- β_1). The middle panel: The same membranes were stripped and re-probed with anti-Smad2 antibody. The bottom panel showed IgG as the loading control.

Discussion

Renal glomerular mesangial cells and tubular cells produce and secrete AM [16-18], which is involved in the regulation of renal function in autocrine/paracrine modes. Studies by us [19] and others [3-6] showed that the plasma concentration of AM increased in a variety of renal diseases. Recently, it was shown that AM is a potent growth inhibitor of renal mesangial cells. Chini et al. [20] reported that AM not only suppressed mesangial cell proliferation stimulated by platelet-derived growth factor (PDGF), epithelial growth factor (EGF) and endothelin-1 (ET-1) in a dose-dependent manner, but also inhibited the generation of reactive oxygen species (ROS) in mesangial cells and infiltrating macrophages. Furthermore, AM induced apoptosis in cultured mesangial cells [21] and inhibited PDGF-BB and angiotensin II-stimulated migration of rat mesangial cells in a dose-dependent manner [22]. In this study, we observed in human renal tubular cells that AM did not inhibit FCS-mediated cell proliferation and did not affect the growth arrest of TGF- β 1. This implicated that the effect of AM on cell growth is cell-specific and cell growth pattern dependent.

Moontuenga et al. [23] recently showed that the expression of TGF- β 1 and AM was regulated in a spatial and temporal manner during rodent embryogenesis. Overlapping patterns of TGF- β 1 and AM expression were observed in several tissues at the same stage of development, or at the same cellular location. This suggested an interaction between TGF- β 1 and AM. Elevated levels of TGF- β 1 protein were detected in renal tubular cells in almost every experimental animal model of renal fibrosis and kidney tissue of various human fibrotic diseases [24]. However, whether AM could influence TGF- β 1-mediated ECM synthesis or degradation has been unknown yet. To our knowledge, this is the first report, demonstrating that AM can decrease ECM biosynthesis in renal tubular epithelial cells induced by TGF- β 1, suggesting modulation of the progression of renal tubulointerstitial fibrosis by AM.

We further tried to address the molecular mechanism of the effects of AM on the action of TGF- β 1. Smad proteins are known to be the most important signal transduction proteins mediating the fibrotic action of TGF- β 1. Upon TGF- β binding to its transmembrane serine/threonine kinases type II receptors, heteromeric receptor complexes of the type I/type II receptor are formed and activated, and subsequently trigger the phosphorylation of the cytoplasmic receptor-regulated

Smads (R-Smads), Smad2 and/or Smad3 [10, 11]. Phosphorylated R-Smads form heteromeric complexes with the common-partner (Co-Smad) Smad4 and are then translocated into the nucleus to regulate transcription of target genes [25, 26]. The third group of Smads, the inhibitory Smads (I-Smads) Smad6 and Smad7, inhibit TGF- β 1 protein signaling [27, 28]. Our results showed that AM had no effect on the level of phosphorylated Smad2, but prevented the down regulation of Smad6 by TGF- β 1. Furthermore, AM can block TGF- β 1-suppressed formation of Smad2/Smad6 complexes. Uchida et al [29] examined the spatial expression of Smad6 and Smad7 mRNA within the kidney, and found that Smad7 mRNA localized only at glomeruli, while Smad6 mRNA was detectable in both glomeruli and tubular cells, suggesting that Smad6 and Smad7 differentially regulate TGF- β signaling in various types of cells. This was confirmed in a recent study showing that Smad6 had no effect on TGF- β signaling in renal podocytes, but in renal mesangial cells [30]. The absence of Smad7 mRNA in renal tubular cells may explain that we could not detect the Smad7 protein in human kidney proximal tubular epithelial cell line HK2, but the role of Smad7 in the kidney fibrosis should be noticed, because overexpression of Smad7 gene in a normal rat kidney tubular epithelial cell line NRK-52E was able to prevent TGF- β mediated tubulointerstitial fibrosis [31]. The mechanism of adrenomedullin interfered with the down-regulation of the expression of Smad6 protein by TGF- β 1 in tubular epithelial cells needs to be further elucidated.

Our study thus shows that AM interferes with TGF- β -mediated increase in the ECM proteins collagen and fibronectin, suggesting that AM may be useful in the treatment of kidney disease. Our data may provide the molecular basis for the beneficial effects of AM observed in vivo, when intravenous infusion of AM improved renal function in patients with chronic heart failure [32], and AM gene therapy attenuated renal damage in hypertensive rats [33]. Those studies together with our results suggest that AM may have pharmaceutical application to attenuate the progression of renal fibrosis.

Acknowledgement

This work was supported by the National Natural Science Foundation of China, No. 30270612 and No.30000079, awarded to Haichang Huang and Taigen Cui, respectively.

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