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Transgenic Expression of an Altered Angiotensin type I AT1 Receptor Resulting in Marked Modulation of Vascular Type I Collagen

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Abstract

The angiotensin II type I receptor (AT1) was modified by replacing its third intracellular loop and C-terminal tail with the corresponding regions from the bradykinin B2 receptor. Transgenic mice were produced that overexpress this mutated receptor (AB3T). Considerably less collagen content in the intact aorta and in primary aortic smooth muscle (aSMCs) cultures was observed in the transgenic mice. On the other hand, elastin content remained unchanged as measured by western blot, and insoluble amino acid quantitation. The contraction of isolated aortas also remained unaltered. The aSMCs derived from the transgenic mice showed a reduction in angiotensin II responsive type I collagen production. In aSMCs from transgenic mice, the cascade of Akt to the mammalian target rapamycin (mTOR) to p70 S6 kinase (p70S6K) was not angiotensin II activated, while in the aSMCs from wild type mice the cascade was angiotensin II activated. Angiotensin activation of Smad2 and Stat3 was also reduced in the AB3T aSMCs. However, no change in the effect of transforming growth factor β (TGF β) on type I collagen production was observed. Also, the activation of ERK and JNK and G protein linked signaling remained unaltered in response to angiotensin II. Akt and PI3K activation inhibitors blocked angiotensin II stimulated type I collagen expression in WT aSMCs, whereas ERK inhibitor had no such effect. Our results point to an Akt/ mTOR/ p70S6K regulation of collagen production by angiotensin II with participation of Smad2 and Stat3 cascades in this process.

Keywords

Angiotensin II; type I collagen; elastin; Akt; transgenic mice

Introduction

Angiotensin II (AngII) is a crucial vasoactive peptide which regulates the physiology of the cardiovascular system generally through its AT1 receptor (Mehta and Griendling, 2007). The AT1 receptor knock-out mouse has demonstrated that this receptor is required for

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vascular and hemodynamic responses to AngII, and that altered expression of the wild type (WT) AT1 receptor gene has marked effects on blood pressure and the strength and elasticity of the blood vessel (Zieman et al., 2005).

We have been working with the angiotensin AT1 receptor and find that we can alter or chimerically exchange specific intracellular regions and thus alter signaling by the receptor (Yu et al., 2007). By exchanging the 3rd intracellular loop and C-tail of the AT1 receptor with the counterparts of the bradykinin B2 receptor, we constructed a mutant AT1 receptor (AB3T) which lost the ability to activate Akt as WT AT1 while continuing to signal through Gαq and Gαi. A transgenic mouse was generated to express this mutant AB3T receptor using a eukaryotic expression vector pCAGGS (with chicken-β-actin promoter and an hCMV immediate early enhancer). During AngII infusion the AB3T transgenic mice responded with a lower blood pressure and decreased cardiac hypertrophy compared to WT (Ahmad et al., 2009).

Acting through its AT1 receptor, AngII is a potent profibrotic effector. For example, a knock-in mouse model with a constitutively expressed gain-of-function mutant of the AT1 receptor demonstrated cardiovascular fibrogenesis (Billet et al., 2007). A study has shown that smooth muscle cells grown from atherosclerotic aortas synthesize more collagen than those from normal aortas (Pietilä and Nikkari, 1980). Increasing evidence has suggested that, at least with regard to effectors such as TGFβ, PI3K and Akt are involved in the modulation of extracellular matrix (ECM) accumulation (Chen et al., 2005; Krepinsky et al., 2005; Kuang et al., 2006; Vittal et al., 2005; Wu et al., 2007). Expression of the AB3T construct is accompanied by a reduced activation of Akt in response to AngII. This provided us with the opportunity to investigate this regulatory action of Akt with respect to angiotensin regulated fibrogenesis.

As illustrated herein, examination of intact aortas and aortic smooth muscle cells isolated from AB3T transgenic mice illustrates that while the isolated aortas retain their capacity for contraction in response to AngII, their ability to produce type I collagen in response to this effector is reduced. Indeed Akt appears to be an important regulator of angiotensin stimulated collagen protein production but not of elastin protein production. Additionally, while the activation of Smad2 by AngII was markedly reduced, the TGFβ ligand itself was not involved. Thus an approach through altered AT1 signal regulation is providing an opportunity to begin to obtain a better understanding of the mechanisms of AngII generated fibrosis.

Materials and Methods

Materials

[³H] Angiotensin II (52.5 Ci/mmol), and [³H] arachidonate (65.9 Ci/mmol) were obtained from Perkin Elmer Life Sciences (Boston, MA). Protease inhibitor cocktail was from Roche Diagnostics (Indianapolis, IN). U0126, wortmanin, and AKT inhibitor II were purchased from EMD Chemicals (Gibbstown, NJ). TGF-β1 was from R&D Systems (Minneapolis, MN). BCA Protein Assay Kit was from Pierce (Rockford, IL). ECL Western Blotting Detection Reagents were from Amersham Biosciences (Piscataway, NJ). The cDNA for Angiotensin II AT1 receptor (pcDNA3.1-AGTR1) was purchased from Missouri S&T cDNA Resource Center (Rolla, MO). The pCAGGS vector was obtained from Dr. Andrew P. McMahon (Harvard Medical School, Boston, MA).

Animals

The transgenic AB3T mice were generated as described (Ahmad et al., 2009). Their genetic background is FVB, obtained from the Jackson Laboratory (Bar Harbor, Maine). The mice

were housed in the animal quarters with 12:12 hours light-dark cycle and all experiments were conducted in the accordance with the “Guidelines for the Care and Use of Animals” approved by the Boston University School of Medicine. The four week old mice were used for all the experiments.

Aortic smooth muscle cell (aSMC) isolation and culture

The ascending and carotid section of the aortas from WT or AB3T transgenic mice were dissected clear of surrounding tissue, stripped of adventitia, and washed in Phosphate buffered saline (PBS) to remove blood. The tissue was then minced in Dulbecco's Modified Eagle Medium (DMEM) (Cellgro, Mediatech, Inc. Manassas, VA) and transferred to a digestion mixture consisting of 500 units/ml collagenase (Sigma C-9891), 2 units/ml elastase (E-0127, Sigma, St. Louis, MO), 1 mg/ml BSA (Sigma A-7888), and 1mg/ml soybean trypsin inhibitor (Invitrogen # 17075-029, Carlsbad, CA); digestion was performed at 37° C for one hour with intermittent agitation. Digestion was stopped by the addition of an equal volume of complete medium consisting of DMEM with 10% fetal bovine serum. Cells were harvested by centrifugation at $400 \times g$ for ten minutes at 5° C, resuspended in complete medium and then passed through a cotton-plugged glass Pasteur pipette twice. The cells were seeded onto collagen-coated T75 flasks (Corning Inc. Corning, NY) in complete medium. Contamination with endothelial cells and (residual) adventitial fibroblasts is minimal because surface cells are preferentially destroyed during the digestion. The confluent aSMCs possess a typical “hills and valleys” morphology. No typical endothelial cell structures were observed. To ensure the purity of the smooth muscle cells in culture, the cells were characterized by immunocytochemistry using anti- α -smooth muscle actin antibody. More than 95 percent of the cells stained positive. The majority of the experiments were conducted with the aSMCs in third passage.

[³H]-AngII ligand binding

AngII binding studies in intact aortic smooth muscle cells were carried out as described previously (Yu et al., 2005). Briefly, confluent cell monolayers in 24-well plates were incubated in binding buffer (50 mM Tris, 120 mM NaCl, 4 mM KCl, 10 μ g/ml bacitracin, 10 mM glucose, 0.1% BSA, 1 mM CaCl₂, 5 mM MgCl₂, 10 mM HEPES pH7.35) containing various concentrations of [³H]-AngII in the absence (total binding) or presence of 1 uM unlabeled AngII (nonspecific binding) for 2 hours at 4°C. Cells were washed three times with ice-cold binding buffer and then solubilized with 0.2% SDS. Radioactivity was determined with a Packard Tri-Carb 1900TR Liquid Scintillation Counter (Packard Inc, Prospect, CT) after addition of 2 ml of Ecolite scintillation fluid (ICN Biomedical, Inc., Aurora, OH). Equilibrium binding data (K_d and B_{max}) were analyzed by best fit to a single site model using the SigmaPlot® 11 program (SPSS Inc., Chicago, IL).

Arachidonic acid release

The arachidonic acid release assay was performed as described previously (Prado et al., 1997). Briefly, the aortic smooth muscle cells were labeled with [³H] arachidonate (0.2 Ci/well) overnight. Cells were then washed three times and incubated with 500 μ l of DMEM containing 2mg/ml bovine serum albumin and incubated with AngII (100 nM) for 20 minutes at 37°C. Medium was collected and centrifuged at $800 \times g$. Radioactivity in the medium was determined in a Packard β counter after addition of 2 ml of Ecolite® liquid scintillation fluid (ICN Biomedical, Inc., Aurora, OH).

Intracellular calcium

The intracellular Ca²⁺ concentration was determined as previously reported (Prado et al., 1998). The aSMCs were trypsinized and washed two times in physiological buffer solution

(140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM glucose, 0.9 mM CaCl₂, 15 mM HEPES, 0.1% BSA). The cells were resuspended at 1.5×10^7 cells/ml and incubated with Fura-2/AM for 30 min (2 μ M final concentration). After 30 min, the cell suspension was diluted 5 times with physiological buffer solution and incubated for another 15 min. Cells were pelleted and resuspended at 1×10^6 cells/ml. The measurement of intracellular Ca²⁺ was then performed using a Hitachi F-2500 Fluorescence Spectrophotometer with FL solutions 2.0 program (Hitachi Inc., Tokyo, Japan).

Real time PCR

Total RNA was extracted from aortic smooth muscle cells using the RNeasy kit (Qiagen, Valencia, CA). RNA was then subjected to reverse transcriptase-PCR using the Superscript III First Strand Synthesis System (Invitrogen, Carlsbad, CA). Negative controls were performed without the reverse transcriptase. The resultant cDNA was used to conduct Taqman real time PCR on the Applied Biosystems 7300 Real time PCR System (Applied Biosystems, Foster City, CA). Taqman reagents for detecting mRNA expression of mouse collagen 1a1 (MA00801666_g1) or GAPDH (MA99999915_g1) and the custom designed Taqman MGB probe(5'-[6-FAM]- CCA GAC GGA GAA GAT -[MGBNFQ] - 3') and primers (Forward: 5'-CAA CGA GAT GAA GAA GTT CAA GGA- 3'; reverse: 5'-AAG CAC AAT CGC CAT AAT TAT CC- 3') for the AB3T receptor were purchased from Applied Biosystems (Foster City, CA). The expression level of AT1 receptor and B2 receptor were measured using the SYBR Green method. The sequences of the primers were as follows: mouse AT1R: forward 5'-GCGTTCAACAGACTGTAGAT-3', reverse 5'-GGGCAG CCATCATCTATTAA -3'; AT2R: forward 5'-CCCTAAAAAGGTGTCCAGCA-3', reverse 5'-CACAGGTCCAAAAAGCCAAT-3'; B2R forward 5'-CTGGGTGTTTGGAGAGGTGT-3', reverse 5'-ACGAGCATCAGGAAGCAG AT-3'. Cycling parameters were as follows: 50°C for 2 minutes, 95°C for 10 minutes, 45 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Results are presented as relative expression normalized to GAPDH and were calculated using the $\Delta\Delta C_t$ method as described in the Applied Biosystems publication, "Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR" (4371095 Rev B).

Western blot analysis

Primary aSMCs isolated from WT and AB3T mice were incubated with 100 nM AngII for 5 min or 24 hours with or without specific inhibitors. The cells were washed three times with ice-cold PBS. Cell lysates were prepared by addition of ice-cold RIPA buffer, 150 mM NaCl, 1.0% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0 (Sigma, St Louis, MO) and complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) and sedimented at 12,000 rpm at 4°C for 20 minutes. The proteins were fractionated on 10% SDS-PAGE gels. Antibodies for detection of phospho-ERK1/2 (Thr202/Tyr204), total-ERK1/2, phospho-JNK, total-JNK, phospho-Akt (Ser473), total-Akt, phospho-mTOR (Ser2448), total-mTOR, phospho-p70 S6 Kinase (Ser371), total-p70 S6 Kinase, phospho-Stat3 (Ser727), total-Stat3, phospho-Smad2 (Ser465/467) and total-Smad2, and GAPDH were purchased from Cell Signaling Technologies (Beverly, MA). Antibodies for type I collagen were obtained from Rockland Immunochemicals Inc (Gilbertsville, PA). The procollagen type 1 has been reported to be visualized as one band when probed by this antibody (Hazra et al., 2004). The (tripo)elastin was detected by the polyclonal rat lung α -elastin (RA75) from Elastin Products Company (Owensville, Missouri). Protein bands were visualized by chemiluminescence and the film scanned with an Epson Perfection 3170 scanner using Epson Scan (version 1.22A) software. The western blot images were analyzed using Sigma Scan10 (Jandel Scientific, San Rafael, CA) to determine the relative intensity of each band.

Isolation and quantitation of insoluble elastin

After extraction of cell layers with guanidine thiocyanate, the resultant insoluble residue was washed with deionized water and incubated in 0.1 N NaOH at 95°C for 45 min. The hot alkali residue was hydrolyzed in 6 N HCl for 24 h at 110°C. Amino acid analysis (Beckman model 6300 with System Gold software; Palo Alto, CA) was used to confirm the characteristic amino acid composition of the elastin, typically consisting of 80% nonpolar amino acids and the characteristic amino acids, desmosine and isodesmosine. Elastin present (in micrograms) was calculated by multiplying the sum (in nanomoles) of all amino acid residues present by 85, the average residue mass.

Transmission electron microscopy and light microscopy

The ascending aortas were obtained following trans-cardiac perfusion with 4.3% glutaraldehyde (Polysciences Inc, Warrington, PA)/0.03 M sodium barbital–sodium acetate buffer (pH 7.4)/ 0.07 M potassium chloride. The samples were then rinsed with 1 X phosphate buffered saline (PBS). Samples for examination with the transmission electron microscope (TEM) were post-fixed in a solution of 1% osmium tetroxide (Ted Pella Inc, Redding, CA) in the buffer, while samples for examination with the light microscope (LM) were not post-fixed. All aortas were rinsed with 1 X PBS, dehydrated, embedded in Araldite 502, and sectioned. For TEM, the Araldite 502 embedded samples were sectioned and stained with uranyl acetate followed by lead citrate (Toselli and Pepe, 1968). For LM H&E staining, the araldite embedded sections were deplasticized as described by Liscia et al (Liscia et al., 1988), stained with Gills #3 hematoxylin (Thermo Scientific, Waltham, MA), rinsed with water, stained with aqueous eosin (Sigma-Aldrich, St. Louis, MO), and mounted with Cytoseal (Richard-Allan Scientific, Kalamazoo, MI). For LM Sirius red (Electron Microscopy Sciences, Hatfield, PA) staining of collagen, deplasticized plastic sections were stained according to the manufacturer's instructions. Additionally, LM collagen visualization, based on the birefringence of collagen, was achieved by employing Sirius red plus polarization microscopy (Junqueira et al., 1979).

Aortic ring contraction

The descending part of the thoracic aorta of the WT and transgenic mouse was isolated, cleared of surrounding tissue including the adventitia and cut into rings 2-3 mm wide for tension recording. The endothelium was removed by rotating the rings around a rough-surfaced needle. The aortic rings were suspended in a vessel bath containing GIBCO® Earle's Balanced Salt Solution (Invitrogen, Carlsbad, CA) at 37°C and bubbled with 95% O₂ and 5% CO₂. Rings were hung with an active tension of 1 gram, and equilibrated for 60 minutes. The rings were first contracted with 1μM phenylephrine to elicit a reference value (100%) in each ring. WT and AB3T rings demonstrated similar contractions in response to phenylephrine. The rings were then exposed to 100 nM AngII. The tension was recorded on Grass FT03 force transducers (Grass Technologies., West Warwick, RI) and collected with a Power Lab acquisition system (ADInstruments, Colorado Springs, CO). Phenylephrine induced contraction was used as 100%. AngII induced contraction was normalized to that percent.

Statistical analysis and data analysis

All results are presented as mean ± standard error. Statistical evaluation of the data was carried out using the student t-test. Probability values less than 0.05 were considered significant.

Results

Expression of the AB3T receptor in the aortic smooth muscle cells

The AB3T receptor, as sketched in supplementary Fig. 1, was generated by replacing the 3rd intracellular loop and C-terminal tail of the wild type angiotensin II type I receptor with the corresponding regions of the bradykinin B2 receptor. This mutant receptor, AB3T, was expressed in transgenic mice via a ubiquitously expressing vector pCAGGS. The AB3T receptor was previously detected in aortic endothelial cells isolated from these mice (Ahmad et al., 2009). In this report we continue to characterize the expression and signaling of this mutant receptor in aortic smooth cells (aSMCs) using the same transgenic model. We conducted real time PCR to compare the expression of selected receptors in the WT and AB3T aSMCs. As shown in supplementary Fig. 2, while there is no significant change in the endogenous wild type (WT) AT1 receptor and bradykinin B2 receptor expression in the aSMCs from AB3T mice, the AB3T mRNA was only expressed in the transgenic aSMCs. At the protein level, expression of the receptors was quantitated with an [³H]-AngII binding assay. As illustrated in Fig. 1, the number of AngII binding sites proved 6.5 fold higher in AB3T aSMCs than in WT aSMCs (WT: 785 ± 17 sites per cell versus AB3T: 5157 ± 72 sites per cell). There was no significant difference in the K_d from WT and AB3T mice (WT: 2.1 ± 0.2 nM vs. AB3T: 2.3 ± 0.1 nM, *p* > 0.1).

Vascular type I collagen and elastin expression

Real time PCR experiments showed reduced α1(I) collagen mRNA levels in the intact aorta and aortic smooth muscle cells from the transgenic mice (Fig. 2). Likewise, basal type I collagen protein expression was decreased in aortic smooth muscle cells from the transgenic mice as detected by western blot (Fig. 3). While elastin mRNA levels were also down in the intact aorta and aortic smooth muscle cells from AB3T mice, the AB3T aSMCs expressed a similar basal protein level of elastin to WT aSMCs. This finding was consistent with the measurement of the insoluble elastin by amino acid analysis. The insoluble elastin level proved similar in the aortic extracellular matrix from WT (2.67 ± 0.65 μg) and transgenic mice (2.75 ± 0.21 μg) (*n* = 5, *p* > 0.1). At the light microscopic level, aorta sections stained by hematoxylin and eosin (H&E) from both strains of mice were indistinguishable. The easily identifiable elastin fibers appeared similar in both vessels also (supplementary Fig. 3). When visualizing collagen deposition by viewing sirius red stained mouse aorta sections under polarized light, we found that the birefringence luminosity of the collagen is decreased in AB3T mouse aortas when compared to WT aortas (Fig. 4). At the electron microscopic level, the elastin fibers from both strains of mice again were indistinguishable, while the collagen bundles appeared less abundant in the AB3T aortas when compared with the WT mouse aortas (Fig. 5).

AngII induced type I collagen and elastin production in the WT and AB3T aSMCs

To better understand the role of AngII in the differential expression of type I collagen and elastin in the blood vessel bed, we investigated its effect in WT and AB3T aSMCs. Western blots show that 24 hour treatment with AngII (100 nM) results in an increase of type I (pro)collagen expression in the WT but only marginally in the AB3T derived cells (Fig. 6 and supplementary Fig. 4). Unlike its effect on type I (pro)collagen production, AngII strongly increased (tropo)elastin protein expression in both WT and AB3T cells (Fig. 6). AngII/AT1 has been reported to induce type I collagen via activation of the TGFβ signaling pathway (Ford et al., 1999; Chen et al., 2004). As illustrated in the same figure, TGFβ induced type I (pro)collagen production and its activation of Smad2 is similar in AB3T aSMCs and the WT SMCs. On the other hand AngII caused Smad2 activation is reduced in the AB3T derived cells.

Kinase activation in the AB3T aSMCs

The phosphorylation of Akt has been linked to increased collagen production particularly via TGF β stimulation (Runyan et al., 2004; Yano et al., 2007). Here we examined the effect of AngII on the activities of Akt and its downstream kinases in conjunction with type I collagen production in smooth muscle cells isolated from WT and AB3T mouse aortas. As found in aortic endothelial cells (Ahmad et al., 2009), AngII effectively increases pAkt levels in the WT but not in the AB3T aSMCs (Fig. 7). As in the aortic endothelial cells (Ahmad et al., 2009) the activation of ERK by angiotensin remains unaltered in the aSMCs from the AB3T mice. Fig. 7 further illustrates that the abrogation of Akt activation is accompanied with reduction in the activation of the downstream mTOR and p70S6K (p70 S6 kinase). A similar lack of activation of Stat3 is observed.

Role of the PI3K/Akt pathway on collagen and elastin production

The signal profiling in the WT and AB3T aSMCs illustrated an association between Akt signaling and type I collagen production. We further examined the effect of the PI3K/Akt pathway on AngII stimulated collagen synthesis by using specific kinase inhibitors. As shown in Fig. 8, the PI3K inhibitor, wortmannin (10 μ M), or Akt inhibitor II (10 μ M) inhibited the AngII (100 nM) induced Akt and mTOR activation. Both inhibitors also reduced AngII stimulated type I (pro)collagen expression. Those inhibitors had similar effects on AngII induced Stat3 activation. On the other hand, the MEK1/2 inhibitor, U0126, inhibited AngII induced ERK1/2 activation but had no effect on type I collagen expression. In the WT aSMCs, (tropo)elastin production in response to AngII was also increased. However, Akt or PI3K inhibitors had no effect on AngII induced elastin synthesis. Interestingly, U0126, an inhibitor of ERK phosphorylation, effectively reduced AngII stimulated (tropo)elastin production

G protein coupled signaling in the aSMCs from AB3T mice

To assess any contribution of G α i linked signaling on the alteration in collagen production, the G α i associated parameter, arachidonic acid release in response to AngII was determined. This signal was retained in the transgenic smooth muscle cells (Fig. 9). In fact the AngII induced arachidonic acid release proved somewhat stronger in the cells from the transgenic mice. Response to bradykinin was used as a comparative control. Clearly, the response to bradykinin remained unchanged in the transgenic mice compared to WT mice. We also measured changes in intracellular calcium concentration in response to AngII as a readout for G α q coupling. A very similar pattern was observed in both cell types. They both mobilized calcium in response to AngII with a slightly stronger response in the cells from the transgenic mice (Fig. 10).

AngII induced aortic ring contraction

To assess direct AngII induced aortic contraction *ex vivo* in the AB3T mice relative to angiotensin induced collagen accumulation, we measured the force produced by aortic rings isolated from the WT and AB3T mice. Despite the slight increase in Ca²⁺ influx in the AB3T aSMCs, the aortas from AB3T mice displayed very similar contraction in response to 100 nM AngII as aortas isolated from the WT mice (Fig. 11). The contractions of the aortic ring in response to phenylephrine remained unchanged in the transgenic rings further illustrating that the general contraction characteristics of the AB3T aorta remain unchanged.

Discussion

Our results demonstrate that angiotensin AT1 receptor signaling can be altered *in vivo* through transgenic overexpression of AT1 mutant cDNA and furthermore that these

alterations can result in signal specific changes. For example, in the case of the AB3T receptor *in vivo* overexpression, AngII activation of G α i linked arachidonic acid release, G α q linked calcium mobilization and phosphorylation of MAP kinases, JNK and ERK remained unchanged. However, in AB3T cells the activation of Akt and the downstream kinases, mTOR, p70 S6K and of Stat3 by AngII was minimal. These signal alterations appear to have important effects on the expression of α 1(I) procollagen mRNA and on type I (pro)collagen protein content of the AB3T aortas and on AngII-driven type I (pro)collagen production in smooth muscle cells derived from the aortas. Both the mRNA and protein levels of type I collagen from intact aortas or isolated aSMCs were decreased considerably in the AB3T transgenic mice. Interestingly, in a knock-in mouse model developed by Billet et al. where the WT AT1 receptor was replaced by a constitutively active AT1 mutant, the major cardiovascular change was peri-vascular and interstitial fibrosis and increased expression of type I collagen (Billet et al., 2007).

The activation of Akt has previously been reported to effect type I collagen production in organs other than the vasculature. For example, Akt enhances Smad3-stimulated α 1(I) collagen mRNA expression in response to TGF β (Runyan et al., 2004). Akt inhibitors reduce AngII induced collagen synthesis in mesangial cells (Yano et al., 2007). Our results, which associate reduced collagen production with loss of Akt activation in the AB3T mice, are intriguing and extend to the involvement of the mTOR/p70S6K in the Akt pathway. We further show here that wortmannin, a PI3K inhibitor and Akt inhibitor II inhibited AngII induced type I collagen synthesis in smooth muscle cells derived from WT mouse aortas. On the other hand, the MEK1/2 inhibitor U0126 had no effect on Akt/mTOR activation or collagen production. The inhibitor studies confirm the causative roles of the Akt axis in AngII regulated type I collagen production. We also associate the transcription factor Stat3 with this regulation. We found that in the WT aSMCs, AngII induced Stat3 activation, but did not do so in aSMC derived from AB3T mice. It has been reported that rats infused long term with AngII exhibited higher levels of activated p-Stat3, collagen synthesis, and atrial fibrosis. The dominant-negative Stat3 abrogated AngII-induced protein synthesis in cultured atrial myocytes (Tsai et al., 2008). It has also been shown that the PI3K inhibitors LY294002 and wortmannin markedly reduce IL-2-triggered Stat3 serine phosphorylation in primary human T cells (Fung et al., 2003). We observed similar inhibitory effect of wortmannin on AngII induced Stat3 activation in the primary mouse aortic smooth muscle cells.

In addition, we found that while AngII increases elastin production, this increase is not regulated through Akt. Inhibitors of Akt and PI3K activation proved ineffective in modulating AngII stimulated elastin expression. Instead, an inhibitor of ERK activation reduced AngII stimulated elastin production. AngII induced ERK activation in the AB3T aSMC remains the same as that found in the WT aSMCs. This could explain why elastin levels in the aSMCs from the AB3T mice remained unchanged. Clearly, signaling changes engendered through the IC3 and C-terminus exchange did not affect the production of elastin at protein level. A surprising observation is that the mRNA level of tropoelastin is decreased in the AB3T aSMCs compared to the WT cells. This indicates that the Akt related signal may regulate the transcription of elastin. This agrees with the finding that the inhibition of Akt phosphorylation abolishes TGF β -induced increases in elastin mRNA expression in lung fibroblast cells (Kuang et al., 2007). The discrepancy between elastin mRNA and protein expression suggests that the regulation of elastin protein may occur during translation. Also noteworthy are the results obtained with TGF β . Our results are showing that the effect of TGF β on procollagen 1a and elastin production didn't change in the aSMCs from the AB3T aortas. This suggests that the TGF β signal pathway in the AB3T aSMCs remains intact. This is also indicated by the continuing Smad2 activation by TGF β in the mutant cells. Therefore, the change of collagen expression in the AB3T mice is not

directly related to TGF β induced signaling. On the other hand, AngII activated Smad2 in the AB3T aSMCs was greatly reduced. These observations suggest that either AngII directly activates Smad2 or activates it downstream of the TGF β ligand, perhaps at the level of the TGF β type I receptor.

Another parameter which remained unchanged in the AB3T mice was the major acute effect of AngII, the contraction of aortic rings in response to AngII. This observation agrees with the intracellular Ca²⁺ concentration results showing that AngII responsive Ca²⁺ translocation remains essentially unaffected in the AB3T mutant receptor.

Thus transgenic overexpression of a mutant AT1 receptor can silence certain aspects of the WT AT1 signaling mechanism. This has been shown previously with other AT1 receptor mutations. For example, Hansel et al. demonstrated that co-expression of a G α q coupling defective mutant of the AT₁ receptor with the WT AT1 receptor abrogated G-protein coupling but preserved ERK activation by AngII (Hansen et al., 2004). The signaling characteristics in our AB3T aortas parallel their findings. These findings suggest that AT1 receptor dimerization is actively involved in forming distinct functional conformations of the AT₁ receptor to mediate its signaling function and that our AB3T mutant receptor is acting in a dominant negative manner.

In sum, transgenic mutant AT1 receptor overexpression which overlays the WT receptor action can be used to study and eventually regulate specific effector signal transmissions *in vivo*. Furthermore, the attenuation of the Akt related signals in the AB3T transgenic mouse provides only a working model to elucidate the mechanism(s) participating in the regulation of fibrogenesis. Mechanisms which lead to uncontrolled fibrosis are far from resolved and in need of much further investigation before meaningful treatment of fibrotic diseases in the cardiovascular/pulmonary systems can be addressed. Our model is providing an approach to better understand these mechanisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. [^3H]-AngII binding assay

Ligand binding experiments were carried out using [^3H]-AngII on wild type and AB3T aortic smooth muscle cells at concentrations ranging from 0.04 to 40 nM. The dissociation constant (K_d) and maximal binding (B_{max}) were calculated using the SigmaPlot® 8 Program Pharmacology Module (SPSS Inc.). Each Point is the average of cells from triplicate wells $\pm$ S.E. from a representative experiment of three experiments. The B_{max} for WT and AB3T was 785 ± 17 and 5157 ± 72 sites per cell respectively and the difference is significant ($p < 0.001$). The K_d for WT and AB3T was : 2.1 ± 0.2 nM and 2.3 ± 0.1 nM respectively. There is no significant difference between the K_d of WT and AB3T cells ($p > 0.1$). The insert is the Scatchard plot of the binding assay.

Figure 2. mRNA levels of $\alpha 1(I)$ collagen and elastin in the intact aorta and aortic smooth muscle cells (aSMCs) from the WT and AB3T TG mice

Results are presented as relative expression normalized to GAPDH and were calculated using the $\Delta\Delta C_t$ method. The mRNA level from AB3T samples was set as 1. $\alpha 1(I)$ collagen and elastin mRNA levels are significantly different between WT and AB3T in the intact aortas and isolated aSMCs. * represents $p < 0.05$ compared with WT.

Figure 3. Type I collagen and elastin protein levels in the aSMCs from the WT and AB3T transgenic mice

A. Western blot analysis of cell lysates (150 μ g total protein) of aortic smooth muscle cells from wild type and AB3T mice. The blot shows the results from the aSMCs from three WT and three AB3T mice respectively. **B.** Bar graph illustrates the quantitation of the blot. The intensity of the bands are shown as normalized to the spot intensity of the WT collagen or elastin. The bar graph shows the average of the three separate samples. * $p < 0.05$ compared with WT.

Figure 4. Light micrographs showing aortas as seen in cross section of the vessel wall
Note aorta adventitial collagen birefringence (arrows) exhibited by Sirius red staining under polar illumination. Original magnification X150.

Figure 5. Transmission electron micrographs showing aortas as seen in cross section of the vessel wall. The arrows indicate the location of collagen bundles. Original magnification X10,000.

Figure 6. Effect of AngII and TGF β on type I (pro)collagen and (tropo)elastin expression and Smad2 activation in the WT and AB3T aSMCs

Confluent aSMCs from WT and AB3T mice were untreated (C) or treated with AngII (100 nM) or TGF β (1 ng/ml) for 24 h. After incubation the whole cell lysates were isolated and analyzed by Western blot. GAPDH was used as a loading control. Because of the strong TGF β induced collagen and elastin expression and Smad2 activation, 30 μ g protein was loaded per sample to demonstrate the effect of AngII and TGF β . Due to this small amount of protein the densities of the WT and AB3T basal bands representing collagen and elastin can not be viewed quantitatively.

Figure 7. Activation of Protein Kinases

A. Mouse aortic smooth muscle cells were incubated with or without 100 nM AngII for 5 minutes. After incubation, the cytosolic extracts were isolated and analyzed by Western blot. GAPDH was used as a loading control. The blots shown are representative of three experiments. **B.** The quantitation of the western blot is illustrated as a graph which shows fold change in spot intensity of the bands with basal level as 1. * $p < 0.05$ compared with WT.

Figure 8. Effects of inhibitors on activation of kinases and type I collagen and elastin levels after incubation with AngII
A. Western blots show the effect of wortmannin, Akt inhibitor II and U0126 on the phosphorylation of Akt, ERK, mTOR, and Stat3 after 5 minutes AngII incubation, and their effect on type I collagen and elastin levels after a 24 hour AngII incubation in aortic smooth muscle cells isolated from WT mice. The blots shown are representatives of three experiments. **B.** The quantitation of the western is illustrated as a graph which shows fold change in spot intensity of the bands with basal level as 1. * $p < 0.05$ compared with AngII alone.

Figure 9. AngII-stimulated arachidonic acid release in WT and AB3T aortic smooth muscle cells
 Data represent the average of triplicate samples from a representative experiment of three separate experiments. AngII induced significantly stronger arachidonic acid release in the AB3T aSMCs than in the WT aSMCs (#, $p < 0.05$). There is no significant difference in bradykinin induced arachidonic acid release between the WT and AB3T aSMCs ($p > 0.05$).
 * $p < 0.05$ the level of arachidonic acid release compared with basal level.

Figure 10. AngII-induced calcium mobilization in WT and AB3T aortic smooth muscle cells
 Calcium mobilization was measured in the aortic smooth cells from WT and AB3T mice as described in Materials and Methods. The results from WT aSMCs is drawn as a black line and from AB3T aSMCs as a gray line. Three separate experiments showed similar results.

Figure 11. AngII-induced contraction in aortic rings from WT and AB3T mice

Rings from WT and AB3T mice were equilibrated with Earle's balanced salt solution for 60 minutes, and contracted with 1 uM phenylephrine to elicit a reference value. The rings were then exposed to 100 nM AngII. Phenylephrine induced contraction was used as 100%. AngII induced contraction was normalized to that percent. There is no significant difference between the contraction of the rings from WT and AB3T mice (WT: 60 ± 17 % of phenylephrine vs. AB3T 58 ± 8 % of phenylephrine, $n = 3$, $p > 0.05$).