Angiotensin II (Ang II) is believed to play a critical role in mediating cardiovascular diseases including hypertension, atherosclerosis and heart failure.\(^1\)\(^,\)\(^2\) Acute stimulation of primary cultured cells such as vascular smooth muscle cells (VSMCs) by synthetic Ang II has been used to elucidate the potential signal transduction mechanisms of the peptide.\(^2\) However, such treatment may not mimic many pathophysiological conditions where the circulating and/or tissue renin–angiotensin systems are chronically upregulated to consistently produce Ang II. As such, consensus of the Ang II mechanisms obtained from cell cultures with the acute stimulation should be confirmed with in vivo animal experiments using chronic Ang II treatment. However, in vivo models are always less flexible in their ability to pinpoint the responsible signaling events.

To fill the gap between these acute and chronic stimulation methods and obtain physiologically relevant signaling information from primary cell culture experiments, we have made an adenovirus vector encoding a furin-cleavable Ang II fusion protein.\(^3\) We found that the adenovirus was able to produce bioactive Ang II constitutively to the culture medium when it was infected in VSMCs. The functional assays further proved the usefulness of this tool to elucidate a relevant signaling mechanism of Ang II in vitro.

**CONCLUSION**

Application of the Ang II adenovirus vector to cultured cells will be useful to elucidate molecular and signaling mechanisms of cardiovascular diseases associated with enhanced Ang II production.

**Keywords:** adenovirus; angiotensin II; blood pressure; gene transfer; hypertension; hypertrophy; signal transduction; the renin–angiotensin system

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**Constitutional Stimulation of Vascular Smooth Muscle Cells by Angiotensin II Derived From an Adenovirus Encoding a Furin-Cleavable Fusion Protein**

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**BACKGROUND**

To fill the gap between acute and chronic stimulation methods of angiotensin II (Ang II) and obtain relevant signaling information, we have made an adenovirus vector encoding a furin-cleavable Ang II fusion protein.

**METHODS**

Vascular smooth muscle cells (VSMCs) were infected with adenovirus to evaluate Ang II production. Also, expression of early growth response-1 (Egr-1) and hypertrophic responses were examined in VSMCs.

**RESULTS**

Acute stimulation of VSMCs with synthetic Ang II showed the peptide had a half-life of less than 1 h. Infection of VSMCs with Ang II adenovirus showed a time-dependent production of Ang II as early as 2 days and up to 7 days postinfection. The Ang II adenovirus induced VSMC hypertrophy, stimulated Egr-1 expression, and suppressed Ang II type 1 receptor mRNA expression. Chronic Ang II infusion in mice for 2 weeks markedly enhanced Egr-1 immunostaining in carotid artery compared with the control saline infusion.

**CONCLUSION**

Application of the Ang II adenovirus vector to cultured cells will be useful to elucidate molecular and signaling mechanisms of cardiovascular diseases associated with enhanced Ang II production.

**Keywords:** adenovirus; angiotensin II; blood pressure; gene transfer; hypertension; hypertrophy; signal transduction; the renin–angiotensin system

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**METHODS**

**Reagents.** Ang II was purchased from Sigma-Aldrich (St Louis, MO). Antibodies for Tyr\(^204\)-phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2), ERK2, and c-Fos were purchased from Santa Cruz Biotechnology (Santa Cruz, CA; sc-7383, sc-154, and sc-52). Antibody for GAPDH was purchased from Millipore (Billerica, MA) (MRB374). Antibody for early growth response-1 (Egr-1) was purchased from Cell Signaling (Danvers, MA; 15F7 4153).

**Cell culture.** VSMCs were prepared from thoracic aorta of male Sprague–Dawley rats (<350 g) by the explant method as described previously.\(^4\) VSMCs were subcultured in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum, penicillin, and streptomycin. Cells from passage 3 to 10 at 80–90% confluency in culture wells were made quiescent by incubation with

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The first three authors contributed equally to the work.

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Adenoviral vectors and infection. An adenoviral vector-encoding furin-cleavable Ang II fusion protein was created using a modified version of the pIgPfsAng II vector\(^3\) as a template which encodes the signal peptide of human prorenin, mouse immunoglobulin heavy chain fragment, a portion of human prorenin prosegment, the furin-cleavage site, and human Ang II\(^1\) (Supplementary Figure S1a online) instead of frog Ang II coded in the pIgPfsAng II vector. The Ang II fusion protein sequence was amplified by PCR and ligated into the pIREs2-enhanced green fluorescent protein vector (Clontech, Mountain View, CA). The fragment containing the fusion protein, internal ribosome entry site and enhanced green fluorescent protein sequences was further ligated into the pENTR4 vector and then recombined into pAd/CMV/5-DEST vector by a reaction with LR Clonase II (Invitrogen, Carlsbad, CA). The control adenoviral vector created is identical to the Ang II-producing vector except it lacks the sequence for the furin-cleavage site and human Ang II. VSMCs were infected with adenovirus except it lacks the sequence for the furin-cleavage site and human Ang II. VSMCs were infected with adenovirus at 100 multiplicity of infection for 48 h, >95% of VSMCs were positive for green fluorescent protein and the control adenovirus at 100 multiplicity of infection for 48 h, >95% of VSMCs were positive for green fluorescent protein and both adenovirus express almost identical amounts of the fusion proteins as confirmed with immunoblotting against mouse immunoglobulin heavy chain (data not shown).

Ang II enzyme immunoassay. Ang II concentration from the media of stimulated or infected cells was determined using an Ang II Enzyme Immunoassay kit (SPI Bio, Montigny le Bretonneux, France) as per manufacturer’s instructions.

Immunoblotting. Immunoblotting was performed as previously described.\(^4\) Quiescent VSMCs grown on 6-well plates were stimulated for specified durations. The reaction was terminated by the replacement of medium with 100 µl of 1x SDS sample buffer. 40 µl of the cell lysates were subjected to SDS-PAGE gel electrophoresis and electrophoretically transferred to a nitrocellulose membrane. The membranes were then exposed to primary antibodies overnight at 4°C. After incubation with the peroxidase linked secondary antibody for 1 h at room temperature, immunoreactive proteins were visualized by a chemiluminescence reaction kit.

qPCR analysis of Ang II receptor mRNAs. Forty eight hours after the adenovirus infection, total RNA was extracted from VSMCs by using TRIzol (Invitrogen) according to the manufacturer’s protocol. The reverse transcription of total RNA to complementary DNA was carried out using the RevertAid First Strand complementary DNA Synthesis Kit (Fermentas, Glen Burnie, MD) in PTC-100 PCR machine at 42°C for 60 min and the reaction was terminated by heating at 70°C for 5 min. The qPCR primers for AT\(_1\)A were determined according to previous report\(^8\) and the primers for ribosomal 18S and AT\(_2\) were designed using the Primer Blast in NCBI. The real-time quantitative PCR was performed with the Maxima SYBG Gene/ROX qPCR Master Mix (2x) kit (Fermentas) in a Perkin-Elmer real-time PCR machine (Mastercycler ep Realplex). The system automatically monitors the binding of a fluorescent dye to double-strand DNA by real-time detection of the fluorescence during each cycle of PCR amplification. The following PCR conditions were used: the first cycle was set at 95°C for 10 min, followed by 40 cycles set at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s with the primers; AT\(_1\): Forward AGGCCCTGGCTTGTGTTTGAG, Reverse: GCTGCCCTGGCCTCTGTC, AT\(_2\): Forward GGGC CCTAAAAGGGTGTCAGCA, Reverse: GCACATCACAGG TCCAAAGAGCCA, Ribosome 18S: Forward CTCTCTTC ACAGGAGGCCTACCG, Reverse: AGGCTATTTTCCGCC GCCCATC.

Hypertrophy assay. To assess Ang II-induced VSMC hypertrophy directly, we measured cell protein accumulation and cell volume,\(^9\) but did not use a radiolabeled leucine incorporation assay in order to avoid unnecessary use of a radioactive compound. Consistent with a highly cited past report,\(^10\) in VSMCs derived from 12 week-old Sprague–Dawley rats, 72h Ang II incubation in serum-free Dulbecco’s modified Eagle medium resulted in increases in cell protein and volume without any significant change in cell proliferation/viability.\(^9\) To measure cell protein accumulation, VSMCs grown on 12-well plates were incubated with serum-free Dulbecco’s modified Eagle medium for 2 days and infected with adenovirus in serum-free Dulbecco’s modified Eagle medium for 3 days or incubated with or without 100 nmol/l Ang II for 3 days. After aspiration of the medium, cells were washed twice with ice-cold Hanks balanced salt solution, and the total amount of cellular protein was measured as previously described.\(^9\) To measure cell volume, after the pretreatments, VSMCs were washed with Hanks balanced salt solution and trypsinized. The cells were then suspended in phosphate-buffered saline and the cell volume was measured by Z2 Coulter Particle Count and Size Analyzer as previously described.\(^9\)

Ang II infusion and immunohistochemistry. All animal procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and Temple University. Eight-week old mixed background control mice\(^11\) were treated for 14 days with either saline or Ang II (1,000 ng/kg/min) via osmotic minipump. The common carotid artery was extracted and fixed. Immunohistochemistry was performed as described previously\(^12\) with Egr-1 antibody.

RESULTS

To assess stability of Ang II in the cultured medium of VSMCs, time-dependent changes of Ang II concentration were measured by an Ang II enzyme immunoassay. Ang II concentration was measured as previously described.\(^9\) To measure cell volume, after the pretreatments, VSMCs were washed with Hanks balanced salt solution and trypsinized. The cells were then suspended in phosphate-buffered saline and the cell volume was measured by Z2 Coulter Particle Count and Size Analyzer as previously described.\(^9\)
Angiotensin II-Producing Viral Vector

To stimulate VSMCs with Ang II chronically, an adenoviral vector targeting Ang II was created. Infection of VSMCs with this adenoviral vector resulted in time-dependent accumulation of Ang II in the culture medium with its peak at day 4 (Figure 1a). Ang II was detectable even at 7 days after the infection. The amount of Ang II generated in the medium correlated with the multiplicity of infection infected to VSMCs (Figure 1b). Cultured medium of VSMCs infected with the control adenovirus encoding the immunoglobulin fragment had no detectable Ang II in any of these time periods.

Acute Ang II stimulation has been shown to downregulate AT₁ receptor proteins as well as AT₁A mRNAs expressed in rat VSMCs, a process known as homologous downregulation. To assess potential alteration of AT₁A mRNA expression by chronic Ang II stimulation via the adenovirus, qPCR analysis was performed. AT₁A mRNA expression was reduced by the Ang II-coding adenovirus compared with the control virus (Supplementary Figure S1c online), whereas AT₂ mRNA was below the detection level by the qPCR in both infection conditions.

To ascertain that the Ang II produced by the adenovirus vector has biological activity, we assessed whether the infection of the Ang II-coding adenovirus stimulated signal transduction events similar to those observed by an acute VSMC stimulation. Compared with the control adenovirus infection, ERK1/2 phosphorylation and Egr-1 induction were enhanced with the Ang II adenovirus, whereas c-Fos amounts remained the same at 48 h after the infection (Figure 1c).

We have demonstrated hypertrophic responses in VSMCs 72 h after an acute Ang II treatment. The Ang II adenovirus but not the control adenovirus increased protein content to similar levels as the acute Ang II stimulation (Figure 1d, left panel). Compared with the control adenovirus, Ang II adenovirus shifts the cell volume distribution curve to the right (Figure 1d, right panel) in a Coulter counter assay confirming the hypertrophic response in the Ang II adenovirus infected VSMCs. To assess whether the in vitro observation with Ang II adenovirus mimics the in vivo condition with enhanced Ang II activity, mice were infused with Ang II or the control saline for 2 weeks. Egr-1 immunostaining in carotid artery was enhanced with the Ang II infusion compared with the control saline infusion (Supplementary Figure S1d online).

**DISCUSSION**

We have created an adenoviral vector to produce Ang II chronically. The amount of Ang II produced can be fine-tuned with multiplicity of infection and the chronic Ang II effects can be maintained for up to 7 days after the infection. In the present study, the range of concentrations of Ang II achieved by the adenovirus was around 50–300 pmol/l, which is comparable to the Ang II concentrations in plasma and tissues in experimental animals in vivo. The maximum (300 pmol/l) concentration is lower than the Ang II concentration required to induce maximum ERK1/2 activation acutely in VSMCs (1–100 nmol/l). However, the Ang II concentration that was maintained during the experimental period was sufficient enough to induce VSMC hypertrophy comparable to the acute Ang II (100 nmol/l) stimulation. Therefore, our Ang II-producing adenovirus vector would be a powerful tool for studying the effects of Ang II in vivo.
may be superior to an acute Ang II stimulation experiment to look for signal transduction events such as those activated by a tissue renin-angiotensin system.

Although a limited number of reports confirmed Egr-1 induction by Ang II,\(^1^9\) Egr-1 has been recognized as a critical transcriptional factor involved in pathological vascular remodeling.\(^1^9\) As such, our findings with chronic Ang II stimulation both \textit{in vitro} and \textit{in vivo} confirm its potential importance in VSMC remodeling associated with enhanced renin-angiotensin system. By contrast, c-Fos which can be induced by acute Ang II treatment\(^2^0\) was not observed with Ang II adenovirus. This is probably due to a transient nature of c-Fos induction. Therefore, c-Fos may not be an appropriate marker for chronic Ang II stimulation.

In conclusion, an adenoviral vector capable of producing Ang II chronically upon infection was created. Further applications of this vector in a variety of experimental systems both \textit{in vitro} and \textit{in vivo} will be expected to advance our knowledge regarding the roles of circulating as well as tissue Ang II in health and diseases.

Supplementary material is linked to the online version of the paper at http://www.nature.com/ajh

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