Sustained Inhibition of Angiotensin I–Converting Enzyme (ACE) Expression and Long-Term Antihypertensive Action by Virally Mediated Delivery of ACE Antisense cDNA

Hongwei Wang, Michael J. Katovich, Craig H. Gelband, Phyllis Y. Reaves, M. Ian Phillips, Mohan K. Raizada

Abstract—Angiotensin I–converting enzyme (ACE) inhibitors have been proven to be highly effective and are for the most part the drugs of choice in the treatment and control of hypertension, congestive heart failure, and left ventricular dysfunction. Despite this, questions regarding side effects and compliance with this traditional pharmacological strategy remain. In view of these observations, coupled with recent advances in gene-transfer technology, our objective in this study was to determine whether the expression of ACE could be controlled on a permanent basis at a genetic level. We argued that the introduction of ACE antisense to inhibit the enzyme would be a prerequisite in considering the antisense gene therapy for the control of hypertension and other related pathological states. Retroviral vectors (LNSV) containing ACE sense (LNSV-ACE-S) and ACE antisense (LNSV-ACE-AS) sequences were constructed and were used in rat pulmonary artery endothelial cells (RPAECs) to determine the feasibility of this approach. Infection of rat RPAECs with LNSV-ACE-S and LNSV-ACE-AS resulted in a robust expression of transcripts corresponding to ACE-S and ACE-AS, respectively, for the duration of these experiments, ie, 8 consecutive passages. The expression of ACE-AS but not of ACE-S was associated with a permanent decrease of ~70% to 75% in ACE expression and a 50% increase in the Bmax for the AT1s. Although angiotensin II caused a concentration-dependent stimulation of intracellular Ca2+ levels in both ACE-S– and ACE-AS–expressing cells, the stimulation was significantly higher in ACE-AS–expressing RPAECs. In vivo experiments demonstrated a prolonged expression of ACE-AS transcripts in cardiovascularly relevant tissues of rats. This was associated with a long-term reduction in blood pressure by ~15 mm Hg, exclusively in the spontaneously hypertensive rat. These observations demonstrate that delivery of ACE-AS by retroviral vector results in a permanent inhibition of ACE and a long-term reduction in high blood pressure in the spontaneously hypertensive rat. (Circ Res. 1999;85:614-622.)

Key Words: virally mediated delivery • angiotensin-converting enzyme inhibitor • endothelial cell • hypertension • gene therapy

The renin-angiotensin system (RAS) plays an important role in the control of blood pressure (BP), body fluid homeostasis, and several other cardiovascular actions.1–3 Hyperactivity of the RAS has been implicated in such pathophysiological states as hypertension, congestive heart failure, left ventricular hypertrophy, and atherosclerosis. The importance of RAS in the pathogenesis of these diseases is further underscored by the fact that pharmacological agents that interrupt either the formation of angiotensin (Ang) II or its actions are a proven therapy for hypertension and many other cardiovascular system–related diseases. Ang I–converting enzyme (ACE) inhibitors, which inhibit the formation of physiologically active Ang II from its inactive precursor, Ang I, are one such class of drugs. These drugs have achieved a widespread usefulness and, for the most part, are the drugs of choice in the treatment and management of hypertension, congestive heart failure, and left ventricular hypertrophy.4,5 The physiological mechanism by which ACE inhibitors produce such diverse benefits is based on the conclusion that ACE is strategically poised to regulate both the RAS and the kallikrein-kinin system. Thus, as a result of ACE inhibition, Ang II formation is blocked, resulting in the reduction of the effects of Ang II on vascular smooth muscles, vascular endothelium, central nervous system, heart, kidney, and other organ systems involved in the regulation of BP and tissue remodeling.4–6 In addition, ACE inhibition promotes bradykinin-mediated stimulation of nitric oxide production, which provides beneficial effects on endothelium, vasodilation, and tissue remodeling.4–6 In summary, ACE inhibitors are a proven pharmacological therapy and appear to exert...
major effects on the vascular endothelium, both to prevent and, in some cases, to reverse the endothelial dysfunction induced by pathological states.

Despite overwhelming evidence in support of effectiveness of ACE inhibitors in the treatment of cardiovascular diseases, these agents are not without major side effects. They are known to cause hypotension (especially in the renin-dependent state of hypertension), hyperkalemia, a reversible decline in renal damage, and cough, to name a few.\(^7\,8\)

For example, cough is a single side effect, which has been associated with the lack of compliance in patients.\(^8\)

The compliance problem is further compounded by the fact that the ACE inhibitors have to be administered on a regular basis for them to provide continuous beneficial effects.

We decided to explore the hypothesis that inhibition of ACE at a genetic level would produce highly specific and long-term antihypertensive effects. This approach, if successful, might resolve the compliance issue and other side effects common with pharmacological therapy. Moreover, it would provide a model to elucidate the cellular and molecular mechanisms associated with the beneficial effects of ACE inhibition in specific Ang II–responsive organs. Antisense cDNA targeted toward ACE for a gene therapy approach offers an additional advantage, given that ACE inhibitors have a proven track record for the treatment of hypertension, and that ACE gene polymorphism has been linked with hypertension.\(^9\,10\)

In this study, we present in vitro and in vivo data demonstrating that antisense action on ACE and antihypertensive effect.

### Materials and Methods

#### Materials

Wistar Kyoto rats (WKY) and SHR bred from our own colony were used for this study. The original source of breeding animal was purchased from Charles River Breeding Laboratories (Wilmington, Mass). Rat pulmonary artery endothelial cells were kindly provided by Gary A. Visner, University of Florida College of Medicine. Amphotropic PA317 packaging cells and NIH3T3 cells were obtained from American Type Culture Collection. DMEM, M199 medium, geneticin (G-418 sulfate), and Superscript II RNase H reverse transcriptase were from Life Technologies. FBS and cadet bovine serum were from BioCell. Trypsin was from Worthington Biochemical Corp. [\(\gamma\)-\(^{32}\)P]dCTP (3000 Ci/\(\mu\)mol) was from DuPont/NEN. Losartan potassium (Dup 753) was a gift from Dr Robert Speth, Washington State University (Pullman, Wash). Oligo(deoxythymidine), dNTP, Taq DNA polymerase, restriction enzyme, T4 ligase, and other cloning reagents were purchased from Fisher Scientific and were of the highest quality available. All of the primers for the PCR reactions were synthesized by the DNA synthesis faculty of the Interdisciplinary Center for Biotechnology Research at the University of Florida.

#### Primer Sequences for Cloning and Detection of ACE-S, ACE-AS, and Endogenous ACE mRNA

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\(\mu\)g indicates nucleotide.

#### Construction of LNSV-ACE-S and LNSV-ACE-AS Retroviral Recombinants

Rat ACE cDNA was generated by RT-PCR with the use of rat ACE-specific primers 1 essentially as described previously.\(^11\,14\)

In brief, the RT reaction was performed with 5 \(\mu\)g of total RNA from rat lung as described previously.\(^11\) Two microliters of RT solution was subjected to PCR with the use of 50 pmol of primers for 30 cycles (94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute). PCR products were electrophoresed, and a DNA band of 938 bp was excised and purified.\(^15\,16\)

The identity of the cDNA was confirmed by sequencing.

#### Recombination of ACE-S and ACE-AS With LNSV

The retroviral vector LNSV was digested with HindIII followed by cloning of ACE-S and ACE-AS sequences corresponding to nucleotides 254 to 1181 in the coding region of the rat ACE into the unique HindIII site by blunt ligation. Recombinant DNA was transformed into competent HB101 bacterial cells, and ACE-S and ACE-AS colonies were selected. The colonies that produced ACE-S or ACE-AS were then grown in Luria Bertani medium with ampicillin (100 \(\mu\)g/mL), and recombinant DNAs were purified. LNSV has a neomycin-resistance gene for selection driven by a long-terminal repeat promoter (Figure 1A).

Various restriction enzymes were used to characterize the recombinants. For example, a SacI digestion of LNSV-ACE-S provided bands corresponding to 

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For ACE-S (Figure 1B), EcoRI digestion provided 3 bands of 

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plasmid (Figure 1A). Final characterization was performed by sequence analysis of all the fragments. The ACE-S sequence cannot and did not encode for active ACE protein, even though it contained the first active site of ACE. This was a result of a deliberate designing of the primers.

**Preparation of Retroviral Medium Containing LNSV-ACE-S and LNSV-ACE-AS Viral Particles**

Exponentially growing PA317 packaging cells in 60-mm-diameter tissue culture dishes were used for transfection and preparation of viral particles. Cells were seeded at 1×10⁶ and were grown for 1 day in DMEM containing 10% FBS. Cells were transfected with 2 μg of recombinant DNA containing LNSV-ACE-S or LNSV-ACE-AS mixed with 2 μL of lipofection in 200 μL of serum-free DMEM. Culture media were replaced 6 hours after transfection, and cells were allowed to grow for 24 hours in DMEM containing 10% FBS. This was followed by G418 (800 μg/mL) selection and extension as described previously. Viral titer for each clone was routinely measured by infecting NIH-3T3 cells.

**In Vitro Experiments**

**Infection of RPAECs With LNSV-ACE-S and LNSV-ACE-AS**

RPAECs grown in monolayers were dissociated by trypsin and plated onto 100-mm-diameter culture dishes at a density of 4×10⁵ cells/dish. Cultures were grown for 3 days in M199 medium containing 10% FBS. Growth medium was replaced with DMEM containing 10% FBS containing 5×10³ colony-forming units (CFU)/mL for LNSV-ACE-S and LNSV-ACE-AS that also included 4 μg/mL Polybrene. Infection was carried out for 12 hours. Medium was removed after infection by M199 with 10% FBS. G418 selection was carried out for 14 days. Cells were subcultured every 7 days in the presence of G418 and used for experiments at confluence, routinely 4 to 5 days after plating.

**Detection of ACE-S and ACE-AS in Infected RPAECs**

RPAECs infected with ACE-S and ACE-AS were grown in 100-mm-diameter culture dishes. One milliliter of Trizol reagent (Life Technologies) was added, and total RNA was isolated according to the protocol supplied by Life Technologies for this product. Total RNA was used for RT-PCR measurement of ACE-S and ACE-AS essentially as described previously. The primers used for the PCR were primers 3 and 4. RNA (5 μg) was subjected to 20 μL of RT solution containing (in mmol/L) Tris-HCl (pH 8.3) 50, KCl 75, MgCl₂ 3, and each dNTP 10; 0.1 mol/L DTT; 0.5 μg of oligo(deoxythymidine) 15; and 200 units of Superscript RNase H reverse transcriptase. The reverse transcription (RT) reaction was run for 50 minutes at 42°C. One microliter of this RT solution was subjected to polymerase chain reaction (PCR) with the use of specific primers for ACE-S and ACE-AS. The total PCR reaction of 50 μL contained (in mmol/L) Tris-HCl (pH 9.0) 20, KCl 10, and MgCl₂ 1.5; 0.1% Triton X-100; 200 μmol/L of each dNTP; 20 pmol of sense and antisense primers; and 2 units of Taq DNA polymerase. The PCR profile was 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute. The PCR products were run on a 1% agarose gel and quantified essentially as described elsewhere.

**RT-PCR Measurement of AT₁, AT₂, and Endogenous ACE mRNAs**

Total RNA from LNSV-ACE-S and LNSV-ACE-AS–infected RPAECs were isolated and subjected to RT and PCR reactions essentially as described above and elsewhere with the use of AT₁, AT₂, and rat ACE primers. Initially, linear relationships between the PCR products and PCR cycles and concentration dependence of the PCR reaction were carried out to establish optimum PCR conditions as described elsewhere. Previously established optimum conditions for AT₁ and AT₂ for and endogenous ACE mRNAs were subsequently used for semiquantification. In brief, 5 μL of PCR samples containing γ-³²P–labeled PCR products were mixed with 5 μL of 2× gel loading buffer (4% Ficoll 400, 20 mmol/L EDTA [pH 8.0], 0.2% SDS, 0.05% bromphenol blue, and 0.05% xylene cyanol). The samples were separated using a 0.75% agarose gel (29:1, acrylamide/bis-acrylamide ratio) in 1× TBE buffer (in mmol/L, Tris base 89, boric acid 89, and EDTA [pH 8.0] 2). The gel was run for 45 minutes at 120 V in a minigel system (Bio-Rad Laboratories).

**Measurement of ACE Activity in LNSV-ACE-S and LNSV-ACE-AS–Infected RPAECs**

Uninfected and LNSV-ACE-S– and LNSV-ACE-AS–infected RPAECs were grown to confluence. Medium was replaced with serum-free M199 for 24 hours. Cells were collected and used to determine levels of ACE activity essentially as described by Neels et al. Briefly, cells were scraped off the dish and homogenized in PBS (pH 7.4) for 10 seconds, followed by centrifugation at 1500g for 10 minutes at 4°C. The supernatants (25 μL) were incubated at 37°C with 25 mmol/L Hip-Gly-Gly substrate in HEPES buffer (pH 7.4) for 40 minutes, and the reaction was stopped by addition of sodium tungstate and dilute sulfuric acid. After centrifugation, borate buffer was used to adjust the pH to 9.6 to deproteinize, and the Gly-Gly generated was analyzed.

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**Figure 1.** A, Map of LNSV-ACE-S and LNSV-ACE-AS. LTR indicates long-terminal repeat; Neo, neomycin resistance gene; and SV40, simian virus 40. Various restriction sites with corresponding nucleotides are represented. B, Restriction enzyme analysis of LNSV-ACE-S and LNSV-ACE-AS. Recombinant DNAs for LNSV-ACE-S and LNSV-ACE-AS were purified and subjected to EcoRI and Sαcl digestion. The bands generated by these 2 enzymes were consistent with the presence of various restriction sites for them and were predicted.
Measurement of $^{125}$I-[Sar$^1$-Ile$^8$]-Ang II Binding to LNSV-ACE-S– and LNSV-ACE-AS–Infected RPAECs

Uninfected and LNSV-ACE-S– and LNSV-ACE-AS–infected RPAECs were established in 35-mm-diameter tissue culture dishes. Cells attached to these dishes were used to measure the binding of $^{125}$I-[Sar$^1$-Ile$^8$]-Ang II to Ang II receptors as described previously.11 In brief, confluent cultures of RPAECs infected with LNSV-ACE-S and LNSV-ACE-AS were rinsed with PBS (pH 7.2) and incubated with 1 mL of PBS (pH 7.2), containing 0.3 nmol/L $^{125}$I-[Sar$^1$-Ile$^8$]-Ang II and 1% BSA, in triplicate for the determination of total binding. In addition, triplicate cultures that also contained increasing concentrations of losartan (1 nmol/L to 10 μmol/L) or PD 123319 (1 nmol/L to 10 μmol/L) were used for competition-inhibition experiments. Binding data were subjected to Scatchard analysis for the determination of $K_d$ and $B_{max}$ values essentially as described previously.11

Measurement of [Ca$^{2+}$], in LNSV-ACE-S– and LNSV-ACE-AS–Infected RPAECs

[Ca$^{2+}$], was measured in nonconfluent RPAECs using epifluorescence microscopy as described previously.22 Briefly, cells were loaded with fura-2 by incubation with 5 μmol/L membrane-permeant fura-2/acetoxymethylester dissolved in 1 mmol/L DMSO and then centered in the optical field of a ×100 oil-immersion fluorescence objective of an inverted microscope (Nikon Diaphot). Before an experiment, the cells were superfused with Tyrode’s solution for 10 minutes to remove excess external fura-2/acetoxymethylester. The superfusion rate was ~2 mL/min. The cells were illuminated alternately with ultraviolet light (10 per second) of 340- and 380-nm wavelength using an IonOptix chopper-based, electronically controlled dual-excitation imaging fluorescence system. Cell fluorescence (emitted light) was collected through a 510-nm barrier filter before acquisition by a photomultiplier tube. Fluorescence signals were digitized online using an IBM-compatible computer and IonOptix fluorescence image acquisition and analysis software. Autofluorescence was minimal, and background images were obtained from a region of the chamber away from the cells to be examined. The fluorescence signals, $F_{340}$ and $F_{380}$, were background subtracted during the experiment.

In Vivo Experiments

Administration of Viral Particles Into WKY and SHR

Five-day-old WKY and SHR were divided into 3 groups: vehicle (control), LNSV-ACE-S (viral control), and LNSV-ACE-AS (experimental). The treatments were injected directly into the left ventricle of the heart under methoxyflurane anesthesia (metofane, Mallinkrod). Treatments consisted of 1 bolus of $5 \times 10^6$ CFU of viral particles in 10 μL of physiological saline and were administered essentially as described previously.23–25 The choice of this route of administration was primarily based on our prior evidence in delivering viral particles conveniently and reproducibly into the vascular system of a 5-day-old rat.23–25 All animals were returned to their respective mothers until weaned. After weaning (~30 days of age) animals were housed individually and were maintained on Richmond Standard laboratory rodent diet and water ad libitum.

Measurement of BP

Indirect systolic BP was measured at a regular interval by the tailcuff method after 35 days of age essentially as described previously.23

Statistical Analysis

All in vitro experiments were carried out with triplicate culture dishes, and each experiment was repeated at least 3 times unless stated otherwise. Thus, each point represents a minimum of 9 dishes from 3 separate experiments. Data are presented as mean±SE, and statistical significance was determined using ANOVA and the Student t test. Semiquantitative analysis of the PCR products was carried out by quantification of relevant bands, assisted by a Bio-Rad Molecular Analysis software. Data were normalized with the use of

![Image](http://circres.ahajournals.org/)

Figure 2. Expression of ACE-S and ACE-AS in LNSV-ACE-S– and LNSV-ACE-AS–infected RPAECs. RPAEC cultures were infected with $5 \times 10^6$ CFU/mL of LNSV-ACE-S (S lanes 1, 3, and 5) or with LNSV-ACE-AS (AS lanes 2, 4, and 6) as described in Materials and Methods. Cultures were subjected to G418 selection for 14 days followed by subculture. This subculture was termed passage 1. After confluence, cells were collected and RNA was isolated and subjected to RT-PCR for the detection of ACE-S and ACE-AS mRNAs in corresponding cells with the use of specific primers as described in Materials and Methods. β-Actin mRNA was used for determination of equal loading and quantification. Top, Representative ethidium bromide–stained gel. Bottom, Mean±SE (n=3).

β-actin mRNA in samples and presented as relative absorbency essentially as described previously.17 Because of the uncertainties involved in the calculation of [Ca$^{2+}$], signals are reported here as a percentage change in the $F_{340}/F_{380}$ ratio. Data are presented as mean±SE, and statistical significance was determined using the Student t test. Indirect BP measurements were performed on 4 to 8 animals per group and analyzed by repeated-measures ANOVA.

Results

Infection of RPAECs With Viral Particles Containing LNSV-ACE-AS

First, a series of experiments were carried out to establish conditions for the infection of RPAECs with LNSV-ACE-AS viral particles. Viral particles containing LNSV-ACE-S have been used as the control throughout the study. Infection followed by the G418 selection process yielded cultures expressing ACE-S and ACE-AS mRNAs in LNSV-ACE-S– or LNSV-ACE-AS–infected cells, respectively. The expression was robust and was maintained for the duration of the experiment, ie, 8 passages (Figure 2). The expression of ACE-S and ACE-AS appeared to be comparable at each
passage, and no significant change in the level of their expression was observed as a function of passage number.

These observations establish that infection with LNSV-ACE-S or LNSV-ACE-AS viral particles resulted in a stable expression of ACE-S and ACE-AS by RPAECs. This appeared to be specific, given that the parent RPAECs that were not infected did not express either ACE-S or ACE-AS (data not shown). Furthermore, other endothelial cells in culture (human retinal endothelial cell) and astroglial cells in primary culture when infected with LNSV-ACE-AS virus specifically expressed the ACE-AS transcript.

Next, we studied the effect of ACE-AS transcript expression on endogenous ACE in RPAECs. Figure 3 depicts characterization of RT-PCR conditions to semiquantify ACE-S and ACE-AS and to determine the effects of ACE-AS expression on endogenous ACE mRNA. Total RNA from RPAECs infected with ACE-S and ACE-AS showed a PCR cycle–dependent linear relationship with the concentrations of endogenous ACE and β-actin mRNAs (Figure 3A and 3B). A linear relationship also was seen between the optical density of the PCR band and the concentration of the mRNAs after RT reactions (Figure 3C). Thus, optimum conditions were used to determine the effect of ACE-S and ACE-AS expression on endogenous ACE mRNA. Expression of ACE-AS caused a 75% decrease in the ACE mRNA when the levels were compared with the parent noninfected endothelial cell cultures (Figure 3D). In contrast, expression of ACE-S showed no significant effect on ACE mRNA. The reduction in ACE-mRNA levels in ACE-AS cells was maintained as the cells were subcultured for 8 passages (Figure 3D). ACE-S–expressing RPAECs showed a specific activity of ACE enzyme of 1.45 mU/mg protein. This was comparable with the level of ACE activity observed in uninfected, parent RPAECs (Figure 4). However, ACE-AS–expressing RPAECs showed a 70% decrease in ACE activity compared with the ACE-S–expressing cells (Figure 4). As for the ACE mRNA, ACE activity was reduced throughout the duration of the experiment (8 passages) in ACE-AS–expressing cells. These observations indicate that infection of RPAECs with LNSV-ACE-AS results in a permanent expression of ACE-AS transcript that is associated with a parallel decrease in transcription of the endogenous ACE gene and ACE activity.

Effects of ACE-AS Expression on Ang II Receptors and Ang II Actions

There are conflicting reports in the literature regarding the effects of ACE inhibitors on Ang II receptor level.26,27
Because ACE inhibitors have diverse effects, not only on the plasma and tissue RAS but also on the bradykinin system, it has been difficult to reconcile these differences. Our in vitro gene delivery system appears to be more selective, given that it would only influence ACE at the genetic level and thus is poised to resolve the issues in a specific manner. With this rationale in mind, we studied the effects of ACE-AS expression on Ang II receptors in RPAECs. Binding of $^{125}$I-[Sar$^1$-Ile$^8$]-Ang II to ACE-AS–expressing RPAECs was 40% to 50% higher compared with ACE-S–expressing cells. Losartan caused a dose-dependent inhibition of this binding in both cell types, although the binding was significantly higher in the ACE-AS–expressing cells at all concentrations of losartan (Figure 5). In contrast, PD-123319, an AT$\textsubscript{2}$ subtype–specific antagonist, failed to compete for $^{125}$I-[binding]. This indicated that RPAECs predominantly express the AT$\textsubscript{1}$ subtype and that the expression of ACE-AS results in an increase in AT$\textsubscript{1}$ binding. This conclusion was confirmed by the Scatchard analysis of the binding data. Table 2 summarizes the observations, which indicate that the increase in the binding in ACE-AS cells resulted from a 50% increase in the B$\textsubscript{max}$ for the AT$\textsubscript{1}$, without a significant change in the K$\textsubscript{d}$ values.

RT-PCR was carried out with the use of specific AT$\textsubscript{1}$ and AT$\textsubscript{2}$ primers to determine whether the increase in the AT$\textsubscript{1}$s occurs at the transcriptional level. Optimum conditions used for this semiquantitative analysis of AT$\textsubscript{1}$ and AT$\textsubscript{2}$ mRNAs have been established previously for cultured cells. Figure 6 shows that AT$\textsubscript{1}$ mRNA levels were predominant in both ACE-S– and ACE-AS–expressing RPAECs. AT$\textsubscript{2}$ mRNA levels, although detectable, were very low. No significant changes in the levels of AT$\textsubscript{1}$ mRNA or AT$\textsubscript{2}$ mRNA were observed between the ACE-AS– and ACE-S–expressing cells. These data led us to conclude that the increase in the AT$\textsubscript{1}$s in ACE-AS cells could be a posttranscriptional event.

Finally, we sought to determine whether the AT$\textsubscript{1}$ in RPAECs is functional and if so, whether an increase in its numbers in ACE-AS cells would be associated with a parallel increase in its function. Because many AT$\textsubscript{1}$–mediated cellular actions of Ang II are linked to the changes in $\left[Ca^{2+}\right]_i$, we studied the effect of ACE-AS expression on Ang II stimulation of $\left[Ca^{2+}\right]_i$. Ang II caused a concentration-
dependent increase in \([Ca^{2+}]_{i}\) in both ACE-S– and ACE-AS–
expressing cells (Figure 7A). The response was maximal, with 100 nmol/L Ang II in both cell types, although the ACE-AS–
expressing cells showed a higher response at each concentration of Ang II compared with ACE-S–expressing RPAECs. Quantification of \([Ca^{2+}]_{i}\) data showed that the levels of \([Ca^{2+}]_{i}\) were significantly higher in Ang-II–treated ACE-AS cells compared with ACE-S cells (Figure 7B). This
was consistent with a higher Bmax for AT1s in these cells. In
contrast to its effect on \([Ca^{2+}]_{i}\), Ang II showed no significant
effects on the incorporation of \([3H]\)thymidine into macromol-
ecules or on cell numbers in both ACE-S– and ACE-AS–
expressing cells.

Effects of ACE-AS on the Development of High
BP in the SHR
Five-day-old WKY and SHR were injected with LNSV-
ACE-S and LNSV-ACE-AS, and indirect BPs were mea-
sured. BP in the SHR was significantly higher than in the
WKY by 60 days of age (152±3 versus 121±3, n=4, 
P<0.01). BPs of LNSV-ACE-S–treated WKY and SHR were
comparable with their respective saline-treated controls at 92
days. However, treatment of SHR with LNSV-ACE-AS resulted in a \(\approx 15\) mm Hg lower BP when compared with
LNSV-ACE-S treatment (Figure 8A). No such reduction in
BP was observed in the LNSV-ACE-AS–treated WKY rats.
Specificity of this antihypertensive effect in the SHR by
LNSV-ACE-AS was further established by treatment with
captopril. Captopril caused a 15- to 18-mm Hg lowering of
BP in the LNSV-ACE-S–treated SHR (Figure 8B). This
treatment showed little effect on BP of LNSV-ACE-AS–
treated SHR.

Various Ang target tissues from 120-day-old SHR that
were treated with LNSV-ACE-S and LNSV-ACE-AS were
harvested to determine whether these prolonged antihyper-
tensive effects are associated with a long-term expression of
ACE-AS in vivo. RNA was prepared and used for RT-PCR
essentially as described previously.\(^{17,31}\) Data in Figure 9 show
that a robust expression of ACE-AS and ACE-S (Figure 9B)
transcripts was seen in lungs, heart, kidney, liver, and spleen.
These observations provide a molecular basis for our physi-
ological data and set the stage to use this technology for
studies on the role of blood and tissue ACE in the control of
hypertension.

Discussion
The most significant observation of this study is that it
demonstrates, for the first time, that a retrovirus based vector
can be used to deliver ACE-AS. This delivery results in a
permanent attenuation of ACE expression and long-term
antihypertensive effects exclusively in the SHR. Thus, the
gene delivery system that inhibits ACE expression at the
genetic level provides a potentially important tool to study the
mechanism by which ACE inhibition causes antihypertensive
actions and produces beneficial effects on the cardiovascular
system.

Infection of RPAECs in culture resulted in the expression
of ACE-S and ACE-AS transcripts. The expression was
maintained throughout the duration of RPAECs in culture, ie,
up to 8 passages. This is equivalent to \(\approx 32\) generations if one
assumes that the doubling time for RPAECs is \(\approx 24\) hours.\(^{32}\) This, coupled with the fact that LNSV is a retroviral
vector and is integrated into the host genome, would indicate
that the permanent expression is a result of integration of ACE-S and ACE-AS into the RPAEC genome. A similar integration was observed when an LNSV vector containing AT1 subtype–specific antisense cDNA was incubated with neuronal and astroglial cells. Expression of ACE-AS was accompanied by a decrease in ACE gene expression as evidenced by decreases in the levels of both ACE mRNA and ACE activity. However, a major difference is that the genetic manipulation is carried out only once to achieve a permanent inhibition of ACE, whereas pharmacological agents have to be constantly provided.

In the RPAECs, inhibition of ACE activity resulted in an increase in the AT1 subtype. These receptors are functional, because their activation was associated with an increase in \([\text{Ca}^{2+}]_i\), and because ACE-AS–expressing cells showed a significantly higher sensitivity to Ang II compared with ACE-S–expressing cells. The increase in the AT1s in ACE-AS–expressing cells is similar to in vivo observations with ACE inhibitors. However, there are other reports that indicate a decrease in AT1s after ACE inhibitor treatment. This conflicting effect of ACE inhibitors would be easily resolved with the use of a gene therapy approach such as the one described here, because it can selectively target Ang-sensitive tissue. The increase in the AT1 was posttranscriptionally regulated, because the mRNA levels for the AT1 did not change between the ACE-AS and ACE-S cells. The mechanism of this control remains to be determined; however, on the basis of our previous observations it is tempting to speculate that the increase could be a result of an increased recycling of AT1s in ACE-AS–treated cells. Thus, an increased mobilization of AT1s to the plasma membrane from the intracellular compartment could be predicted. This conclusion is based on the fact that significant levels of intracellular AT1s are shown to be present in a wide variety of cells.

Why should one attempt to develop the means to inhibit ACE at a genetic level when proven pharmacological agents are available to do so? The question is particularly relevant, because ACE inhibitors are highly successful in the treatment of hypertension, congestive heart failure, and other related diseases. The rationale for a genetic approach includes the following: (1) ACE inhibitors need to be administered on a regular basis to maintain their beneficial effects, whereas the gene therapy approach could potentially be accomplished with one treatment; (2) ACE inhibitors have significant side effects; (3) compliance appears to be an important impediment in any traditional pharmacological strategy, particularly when it is associated with significant side effects; (4) it has been difficult to elucidate the precise mechanism of action of ACE inhibitors because of their diverse effects on the cardiovascular system, especially the kinin system, whereas gene transfer could produce organ-specific targets for ACE inhibition, which could be used to evaluate the mechanism; and (5) genetic intervention holds the potential for both the long-term control and the prevention of hypertension, given that ACE polymorphism is associated with hypertension. Thus, targeting ACE to investigate its potential in the use of gene therapy for the control of hypertension seems to be a rational approach. This is particularly important in view of well-established pharmacological literature indicating that ACE inhibition improves endothelial dysfunction, controls hypertension, and improves vascular remodeling, among many other beneficial effects. In fact, our in vivo data support this contention.

Delivery of ACE-AS into rats caused a long-term expression of ACE-AS transcript in various Ang-target tissues. This was associated with a significant attenuation of high BP, which was exclusive for the SHR. The fact that captopril failed to significantly reduce BP in the ACE-AS–treated SHR clearly indicated that the LNSV-ACE-AS strategy is targeting the expression of endogenous ACE. These observations provide preliminary evidence for the usefulness of this system to study the precise physiological mechanism of antihypertensive effects of ACE.
inhibition. It also sets the stage to determine the involvement of the bradykinin-nitric oxide system in the beneficial effects of ACE inhibitors on cardiovascular system.

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References

Sustained Inhibition of Angiotensin I–Converting Enzyme (ACE) Expression and Long-Term Antihypertensive Action by Virally Mediated Delivery of ACE Antisense cDNA

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