In Vitro Evidence for an Intracellular Site of Angiotensin Action

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Abstract—To differentiate the relative effects of nuclear and cell surface angiotensin II (Ang II) receptors, we mutated the angiotensinogen cDNA by removing the signal sequence-encoding region to produce a nonsecreted form of angiotensinogen [Ang(−S)Exp]. Rat hepatoma cells (which produce renin and angiotensin-converting enzyme mRNAs) were stably transfected with Ang(−S)Exp/pSVL (or a corresponding control) expression plasmid, and mitotic indices were measured for stably transfected cell lines. Experimental clonal cell lines demonstrate an average of 33±4.4% (P<0.001) increase in percentage-labeled nuclei compared with control cell lines. The mitogenic effect is blocked by 10−6 mol/L losartan and by 1 μmol/L renin antisense phosphorothioate oligomers but not by 10−6 mol/L candesartan. In addition, phenylarsine oxide, which blocks angiotensin receptor internalization, abolishes the losartan inhibitory effect, suggesting that after cell-surface receptor-mediated endocytosis, losartan blocks Ang II nuclear receptors. PDGF mRNA levels are elevated 2.2-fold in Ang(−S)Exp transfected cell lines; addition of anti-PDGF antibodies to the culture medium partially blocks the mitogenic effect of Ang(−S)Exp, while anti–Ang II antibodies have no effect. These results suggest that the Ang(−S)Exp growth effect is due, in part, to autocrine/paracrine stimulation by secreted PDGF after Ang II/Ang II receptor intracellular interactions. We further demonstrate that these cells produce the alternative renin transcript, renin 1A, which apparently lacks a signal sequence and is maintained intracellularly. Collectively, these studies of cultured cells suggest that some cell types may possess components of the renin-angiotensin system that permit intracellular processing of angiotensinogen to Ang II and that Ang II generated intracellularly may be mitogenic. (Circ Res. 2001;89:1138-1146.)

Key Words: angiotensinogen ■ angiotensin ■ intracrine ■ intracellular ■ receptor blocker

The existence of nuclear angiotensin II (Ang II) receptors has been demonstrated by a number of laboratories.1–4 Re and colleagues1 showed that isolated rat liver and spleen nuclei specifically bound 125I-labeled Ang II with high affinity. In these studies, Ang II competed effectively for radio-labeled tracer whereas Ang I did so less effectively and neurotensin not at all. Their studies extended earlier investigations that suggested that labeled Ang II localized to nuclear and mitochondrial regions of myocardial, brain, and smooth muscle cells.5–7 Re and colleagues further demonstrated 125I–Ang II binding to solubilized rat liver chromatin fragments, the existence of a discrete Ang II–binding nucleoprotein particle by deoxynucleoprotein gel electrophoresis, and direct effects of nuclear angiotensin on transcription.2,8,9 In addition, Ang II immunoreactivity has been found associated specifically with euchromatin in cerebellum, liver, and adrenal tissue.10 Erdmann and colleagues,10 using immunogold staining, found Ang II immunoreactivity to be prominent in cerebellar neurons; the peptide was localized to nuclei and also to vesicle-like structures in cytoplasm. In some cell types such as endothelial and granule cells, the peptide was nearly exclusively present in the transcriptionally active euchromatin leading the authors to suggest that Ang II directly regulates gene expression.

Baker and colleagues4 further characterized the kinetics of binding of Ang II (versus competitors) to nuclei and nuclear envelopes, and showed that inhibitors of AT1–Ang II binding (losartan) also inhibited Ang II–nuclear receptor interactions. Dzau and colleagues3 proceeded to (partially) characterize the Ang II nuclear receptor as being AT1-like (similar in size, losartan-inhibited) but distinct with respect to several physico-chemical properties.

Similarly, Egunna and colleagues11,12 have shown that rat hepatic nuclei demonstrate Ang II receptors (about 10% of which are associated with chromatin) that are AT1-like; losartan behaves as an effective Ang II antagonist for these nuclear receptors. In addition, they identified specific transcriptional changes associated with Ang II treatment of isolated rat hepatic nuclei. c-Myc, platelet-derived growth factor (PDGF), renin, and angiotensinogen (Aogen) mRNA

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levels are all increased (as determined by nuclear run-on assays), in a biphase dose-dependent fashion, after Ang II exposure. Jimenez et al. further characterized Ang II receptors from rat liver nuclei using selective ligands for \( \text{AT}_1 \) and \( \text{AT}_2 \) subtypes in radioligand binding and isoelectric focusing assays. They found adult rat liver cells to possess only \( \text{AT}_1 \) receptors although isoelectric focusing profiles revealed two Ang II–receptor complex peaks (pI \( = 6.5 \) and 6.8) for plasma membrane and one peak (pI \( = 6.8 \)) for nuclear preparations. They suggest that this charge heterogeneity could account for the distinct physicochemical properties of nuclear versus plasma membrane receptors previously detected. They also suggest that posttranslational modifications could accompany receptor endocytosis and recycling and could account for the charge variability. They further showed that monensin, which disrupts Golgi trafficking, does inhibit nuclear accumulation of the labeled peptide suggesting that Golgi bodies might be involved in Ang II nuclear accumulation.

Raizada and colleagues showed that Ang II–induced chronic neuromodulatory stimulation of rat primary neuronal cultures can lead to nuclear sequestration of the \( \text{AT}_1 \) receptor, presumably involving the putative nuclear localization signal within the cytoplasmic tail of the receptor. Because other studies suggest that the nuclear receptor is distinct from the plasma membrane receptor, it is possible that multiple receptors or multiple forms of the same receptor can reside in the nucleus depending on cell type and condition.

Collectively, these studies suggest that Ang II may be internalized or generated through an intracrine system and that it alters cellular properties after nuclear translocation, receptor binding, and transcriptional regulation of gene expression. Furthermore, the nuclear receptor is likely identical to, or distinct from but quite similar to, the plasma membrane \( \text{AT}_1 \) receptor.

To investigate the potential for (and subsequent effect of) Ang II generation within cells, we mutated an Aogen cDNA and ligated it into an expression plasmid to produce a nonsecreted (−S) form of Aogen. Ang II produced from this clone with a mutated Ang I domain. Signal peptide extends from M (methionine at amino acid position 1) to G (glycine at amino acid position 24). B, Primers used to generate Ang(−S)Exp and Ang(−S)Cntr products. The ATG translation start site is boxed. CS indicates consensus sequence. Ang(−S)Exp and Ang(−S)Cntr were cloned into pSVL to generate expression vectors Ang(−S)Exp/pSVL and Ang(−S)Cntr/pSVL, respectively.

### Materials and Methods

#### Preparation and Cloning of Mutated Aogen cDNA
Reverse transcription–polymerase chain reaction (RT-PCR) was conducted as indicated below using the Access RT-PCR System (Promega) and H4-II-E-C3 rat hepatoma RNA. Angiotensinogen upstream primers [Ang(−S)U1] and [Ang(−S)U2] were each used with Ang(−S)D (downstream primer) to generate PCR amplification products Ang(−S)Exp and a corresponding control, Ang(−S)Cntr, respectively (Figure 1A). Each of the upstream primers is partially template-complementary. The noncomplementary extension of each contains an ATG translation start site, a Kozak consensus sequence to enhance ribosome association and translation, and a XhoI restriction endonuclease recognition site for cloning (Figure 1B). The downstream primer extension possesses a XhoI restriction endonuclease recognition site for cloning. The PCR products contain the full-length 3′-untranslated region including mRNA stabilizing sequences (≈1.6 kb).
The RT-PCR product was ligated into pCR II from the Invitrogen Original TA Cloning Kit. INVVo' cells were transformed, selected with X-Gal (Gibco/BRL), and tested for inserts. Inserts were then removed by digestion with XhoI, ligated into XhoI-digested pSVL (SV40 late promoter, VP1 intron), and orientation determined by restriction digestion with NcoI. Dixoxy sequencing (primer, pSVL, 5'-GGAAATGTATCCTCTGCTC-3') that we designed complementary to pSVL plasmid sequence) was used to confirm fidelity of the Aogen cDNA PCR mutagenesis.

### Primer Extension

Primer extension was used to confirm expression of the transgenic clone mRNAs [Ang(-S)Exp and Ang(-S)Cntr]. The artificial sites introduced via primers during PCR afford unique hybridization sites for primer hybridization and extension.

#### Ang(-S)Exp/pSVL

(coding DNA) 3'-CTAGCTGAGCTCCGGTGGTACCAT...-5' mRNA 5'-GAUCGACUCGGACCAUCUUAGC-...3' primer 3'-CTAGCTGAGCTCCGGTGGTACCTG-5'.

#### Ang(-S)Cntr/pSVL

(coding DNA) 3'-CTAGCTGAGCTCCGGTGGTACCAT...-5' mRNA 5'-GAUCGACUCGGACCAUCUUAGC-...3' primer 3'-CTAGCTGAGCTCCGGTGGTACCAT-5'.

The control primer for both primer extension reactions is a scrambled version of that used to extend the Ang(-S)Exp product (3'-ATTCGGAGCTCCTGCTGACCAT-5').

Oligonucleotide primers were labeled using T4 polynucleotide kinase to a specific activity of at least 10^8 cpm/µL. RNA (10 µg) was hybridized to 2 µL of labeled primer in 0.4 mol/L NaCl, 10 mmol/L Pipes, pH 6.4 (volume of 10 µL) overnight at 30°C. Annealed primer/RNA complexes were then added by extension of 90 µL of extension buffer. Mix (per 90 µL): 5 µL 1 mol/L Tris-Cl, pH 8.2; 5 µL 0.2 mol/L dithiothreitol (DTT); 5 µL 0.12 mol/L MgCl2; 2.5 µL 1 mg/mL actinomycin D in water, 5 µL 10 mmol/L dNTPs (nucleotides from Pharmacia); 25 µL of AMV reverse transcriptase (Promega); water.

Products were electrophoresed on an 8% denaturing polyacrylamide gel. M13 mp18 single-strand DNA was sequenced with the -40 primer using the T7 Sequenase 7-deaza-dGTP sequencing kit (USB Corp).

### Transient Transfections

H4-II-E-C3 rat hepatoma cells were plated in EMEM+10% FBS at 10^4 well per 12-well plate and permitted to attach overnight prior to transfection. Cells were transfected, 4 wells per clone, with Ang(-S)Exp/pSVL or Ang(-S)Cntr/pSVL plasmids (0.5 µg DNA and 3 µg of lipofectin (Gibco/BRL) per well) for 4 hours. Media were replaced at 24 hours with addition of fresh drug. At 48 hours, BrdU-labeling was initiated and proceeded for 3 to 6 hours prior to staining.

### Establishment of Stable Transfectants

H4-II-E-C3 rat hepatoma cells were plated at 1.5×10^5 per 60-mm dish and permitted to attach overnight prior to transfection. Cells were transfected with Ang(-S)Exp/pSVL or Ang(-S)Cntr/pSVL (10 µg), pSV2neo^15 selectable marker plasmid (1.0 µg) and lipofectin (30 µg). Forty-eight hours after transfection, G418 (Calbiochem) selection was initiated (400 µg/mL) and stable transfectants were isolated by limited dilution.

### Analysis of Clonal Isolates

H4-II-E-C3 rat hepatoma transfected cell lines were plated on 96 well plates at 5000/well and permitted to attach overnight. Where indicated, Ang II, losartan, and/or candesartan CV-11974 (each to 10^-4 mol/L), phenylarsine oxide (PAO) at 20 µMol/L, and/or DT to 1 mmol/L, were added to the BrdU labeling medium for a 3- to 6-hour incubation period. For wells receiving both candesartan and losartan, candesartan was added to 10^-5 mol/L and incubated for 30 minutes. Losartan was then added to 10^-6 mol/L (to the medium containing the candesartan). All experiments were repeated 3 to 4 times. For wells receiving both PAO and candesartan, losartan, or Ang II, PAO was added for a 5’ incubation prior to the addition of the other substance. For wells receiving PAO and DT, the compounds were added simultaneously.

We determined the ED50 (effective dose, 50%) for H4-II-E-C3 cells to be 3.7 mg/mL PDGF AA (Sigma) (data not shown) with a maximum response at ~25 ng/mL. The corresponding ND50 (neutralization dose, 50%) (for PDGF AA neutralizing antibody, R&D Systems) at the PDGF maximum response dose was 0.17 µg/mL. Complete neutralization occurs at 1 µg/mL. The antibody was, therefore, used at 5 µg/mL. An anti-human PDGF-AA neutralizing antibody was used that has been shown to cross-react with rat PDGF.16 For the anti-Ang II antibody (Peninsula Laboratories, Inc, San Carlos Calif, rabbit anti-human angiotensin II), we determined that 7.5 ng of antibody per 100 µL volume of medium was required to neutralize Ang II at a 10^-5 mol/L concentration. We used a maximum of 50-fold the theoretical neutralization dose or 3.75 µg/mL.

### BrdU Labeling and Staining

BrdU (10 µmol/L) labeling was initiated and labeling proceeded for 3 to 6 hours (based on our pilot studies, the optimum labeling time for these experiments). The labeling reagent was removed, and anti-BrdU staining was conducted using the Oncogene Research Products BrdU kit. Approximately 100 cells/field×10 fields were counted (1000 for each treatment) by a technician blinded to the study identifiers.

### Northern Blot

RNAs were harvested by the method of Chomczynski and Sacchi.17 Ten micrograms of each RNA was electrophoresed on a denaturing agarose gel and transferred to Zeta- Probe blotting membrane (Bio-Rad Laboratories). The membrane was sequentially probed with sequences from mouse PDGF (~900 bp EcoRI fragment) and rat ribosomal S3 protein cDNA.18 cDNA inserts were gel-eluted and radiolabeled using the Prime-It II kit from Stratagene. Filters were hybridized with 1 to 2×10^5 cpm/mL (~10^9 cpm/µg).

### RT-PCR for Renin and ACE

RT-PCR was performed using the Access RT-PCR System (Promega) to confirm the presence of angiotensin-converting enzyme (ACE) and renin RNAs in H4-II-E-C3 rat liver cells. A single reaction mix was prepared containing AMV/Taq reaction buffer (1X), dNTPs (0.2 mmol/L), primers (1 µmol/L), MgSO4 (1 mmol/L), AMV RT (0.1 µL/L, Taq DNA polymerase (0.1 µL/L), and RNA sample (1 µg) or kit control. Mixture was incubated at 48°C for 45 minutes in a water bath. PCR reaction conditions were as follows: 94°C, 2 minutes)×1 cycle, 94°C, 30 seconds; 60°C, 1 minute; 68°C, 2 minutes×40 cycles, 68°C, 7 minutes×1 cycle.

### Establishment and Staining

RT-PCR primers are as follows:

- Upstream: 5'-TCAGATGCGCTGGATGCCAG-3' (exon 2)
- Downstream: 5'-GATCCCATGTTGATGGTCAA-3' (exon 4)

Amplification product size is 223 bp.

### Analysis of Clonal Isolates

H4-II-E-C3 rat hepatoma transfected cell lines were plated on 96 well plates at 5000/well and permitted to attach overnight. Where indicated, Ang II, losartan, and/or candesartan CV-11974 (each to 10^-4 mol/L), phenylarsine oxide (PAO) at 20 µMol/L, and/or DT to 1 mmol/L, were added to the BrdU labeling medium for a 3- to 6-hour incubation period. For wells receiving both candesartan and losartan, candesartan was added to 10^-5 mol/L and incubated for 30 minutes. Losartan was then added to 10^-6 mol/L (to the medium containing the candesartan). All experiments were repeated 3 to 4 times. For wells receiving both PAO and candesartan, losartan, or Ang II, PAO was added for a 5’ incubation prior to the addition of the other substance. For wells receiving PAO and DT, the compounds were added simultaneously.

We determined the ED50 (effective dose, 50%) for H4-II-E-C3 cells to be 3.7 mg/mL PDGF AA (Sigma) (data not shown) with a maximum response at ~25 ng/mL. The corresponding ND50 (neutralization dose, 50%) (for PDGF AA neutralizing antibody, R&D Systems) at the PDGF maximum response dose was 0.17 µg/mL. Complete neutralization occurs at 1 µg/mL. The antibody was, therefore, used at 5 µg/mL. An anti-human PDGF-AA neutralizing antibody was used that has been shown to cross-react with rat PDGF.16 For the anti-Ang II antibody (Peninsula Laboratories, Inc, San Carlos Calif, rabbit anti-human angiotensin II), we determined that 7.5 ng of antibody per 100 µL volume of medium was required to neutralize Ang II at a 10^-5 mol/L concentration. We used a maximum of 50-fold the theoretical neutralization dose or 3.75 µg/mL.
Identification of Rat Renin 1A Transcript in H4-II-E-C3 Cells

To determine whether H4-II-E-C3 cells possess renin 1A mRNA, RT/PCR of H4-II-E-C3 RNA followed by agarose gel electrophoresis, cloning, and sequencing were performed. Specifically, renin 1A cDNA was amplified using an upstream primer designed to hybridize within exon 1A (5′-TCAGCGGGCCGTGTCCAAA-3′) with a downstream primer complementary to exon 2 sequences (5′-AAAGTATGTGGAGACCACA-3′). (Exon 1A is derived from intron 1 of the rat renin primary transcript.) The Promega Access RT-PCR Introductory System Kit was employed following the manufacturer’s recommendations. PCR conditions were [94°C, 2 minutes] × 1 cycle, [94°C, 30 seconds; 60°C, 1 minute; 68°C, 2 minutes] × 40 cycles, [68°C, 7 minutes] × 1 cycle. PCR products were ligated into PCR 2.1 from the Invitrogen Original TA Cloning Kit and sequenced using the M13 reverse primer and the Sequenase 7-deaza-dGTP DNA Sequencing Kit (USB Corp). Six different primer pairs designed to hybridize to exons 1 and 2, and thus to PCR amplify the classical preprorenin mRNA, produced no product suggesting, that these cells make only (the alternative) renin 1A mRNA.

Antisense Oligonucleotide Treatments

Ang(−S)Exp/pSVL Clone 1 and naive H4-II-E-C3 cells were treated with 1 μmol/L antisense or scrambled control phosphorothioate oligonucleotides (5′-TGCTGAATGGGCTGTTCAAA-3′ and 5′-TGCTAGATGGAGATTTACA-3′, respectively) for rat renin mRNA. A BLAST search of candidate antisense sequences (www.ncbi.nlm.nih.gov/) was conducted to avoid undesirable matches to mammalian DNA within GenBank. Cells were treated with oligonucleotides for 36 hours with one change of medium, after which BrdU labeling was performed for 6 hours (oligomers were maintained) and cells stained.

Statistics

Groups were compared using a one-way analysis of variance (ANOVA) with Tukey-Kramer or Bonferroni multiple comparisons post hoc tests.

Results

Prime Extensions

Primer extensions were performed using RNA derived from naive H4-II-E-C3 cells, clones 1 and 2 (Ang(−S)Exp/pSVL), and clones 1 and 2 (Ang(−S)Cntr/pSVL) (Figure 2). (See following section for description of clone generation and isolation.) Primers were designed complementary to the unique extensions generated by the PCR amplification primers such that they hybridize only to the transgenes and not to the endogenous Aogen gene. PCR amplification products of the predicted size (124 bp) were generated from RNA of each transgenic clone but not from RNA of naive cells.

Transfections, Transient Assays, and Stable Transgenic Lines

Ang(−S)Exp/pSVL and Ang(−S)Cntr/pSVL were transiently transfected into H4-II-E-C3 cells and mitotic indices measured at 48 hours after transfection. Ang(−S)Exp/pSVL consistently increased nuclear labeling by approximately 20% (P < 0.05) in 6 separate experiments, the increases of which were losartan-inhibited (Figure 3A). The control plasmid, Ang(−S)Cntr/pSVL, had no effect on nuclear labeling. The plasmids, either Ang(−S)Exp/pSVL or Ang(−S)Cntr/pSVL, were transfected into H4-II-E-C3 cells together with a plasmid bearing the neomycin phosphotransferase-selectable marker gene (pSV2-neo). Following G418 selection for several weeks, stably transfected cell lines were isolated and proliferation rates were determined by BrdU nuclear labeling. Measurements were made for 4 clones of each plasmid construct (clones 1 to 4) (Figure 3B). Stably integrated Ang(−S)Exp/pSVL increased labeling an average of 33% (P < 0.001) over Ang(−S)Cntr/pSVL. In each of Ang(−S)Exp/pSVL clones 1 to 4 proliferation was losartan-inhibited resulting in a 25% average decrease in nuclear labeling (P < 0.01). Proliferation of Ang(−S)Cntr/pSVL clones 1 to 4 was not losartan-inhibited.

Candesartan Studies

Our early transfection experiments demonstrating losartan-inhibition of Ang(−S)Exp-induced proliferation (in cells assayed at 48 hours after transfection and in stably transfected clones) was surprising to us. We expected losartan to inhibit only exogenous Ang II, not internally generated and retained Ang II. Therefore, we proceeded to test a second angiotensin receptor blocker, candesartan CV-11974 (gift of AstraZeneca, Sweden). Candesartan did not inhibit the growth of Ang(−S)Exp/pSVL clone 1 (Figure 3C). A recent study from another laboratory suggests that the tight-binding and acid-resistant retention properties of candesartan when associated with AT1 receptor are independent of receptor internalization. Candesartan/receptor complexes appear to remain surface-bound. If candesartan traps receptor at the cell surface and inhibits receptor/ligand internalization and recycling, we reasoned that pretreatment of cells with candesartan would prevent losartan-inhibition of Ang(−S)Exp-stimulated growth. Indeed, pretreatment of cells for 30 minutes with
candesartan followed by losartan addition for 6 hours (during BrdU labeling) eliminated the losartan-inhibition (Figure 4A) of proliferation. Our additional studies showed that candesartan and losartan (10^{-6} \text{ mol/L}) were equally effective in eliminating the mitogenic effect of Ang II added directly to the media of naive H4-II-E-C3 cells (Figure 4B). This demonstrates that both drugs are equally effective in competing with Ang II/AT1 receptor interactions on the cell surface and provides further evidence that the difference between the efficacy of these two receptor blockers for the transfected cells is due to intracellular Ang II generation and cellular retention.

**Inhibitors: PAO, Anti-PDGF Antibodies, Anti-Ang II Antibodies**

Phenylarsine oxide (PAO) has been used in a number of studies^{20–24} to inhibit Ang II receptor endocytosis in a DTT-sensitive fashion. In the present study, 20 \(\mu\)mol/L PAO administered prior to losartan addition to the culture medium blocks the losartan antimitogenic effect on Ang(−S)Exp/pSVL transfected cells. DTT (1 mmol/L) reverses the PAO effect (Figure 5A). Neither PAO or DTT, used in isolation, has growth effects on Ang(−S)Exp/pSVL Clone 1. Similarly, neither PAO or DTT affect growth of H4-II-E-C3 naive cells (Figure 5B). However, PAO blocks the mitogenic effect of exogenously added Ang II (presumably by blocking receptor recycling).

Anti–Ang II antibodies (used at 50-fold the theoretical dose required to neutralize 10^{-6} \text{ mol/L} exogenously added Ang II) completely suppressed exogenous Ang II–induced proliferation of H4-II-E-C3 naive cells (Figure 5B) but failed to inhibit growth of Ang(−S)Exp/pSVL clone 1 (Figure 5A), further suggesting that Ang II generated by the transgene is not exported and does not act through an extracellular mechanism. Conversely, anti-PDGF antibodies significantly reduce growth of Ang(−S)Exp/pSVL Clone 1 and effectively eliminate the mitogenic effect of PDGF (25 ng/mL) on H4-II-E-C3 naive cells (Figure 5B).

**Northern Blot Analyses**

Northern blot analyses (Figure 6) of stably transfected independent clones (3 each construct) indicate that PDGF steady-state mRNA levels are elevated 2.2-fold in Ang(−S)Exp/pSVL compared with Ang(−S)Cntr/pSVL clones. Collectively, with the anti-PDGF antibody treatment results, this suggests that the mitogenic effect of Ang(−S)Exp (and thus the mitogenic effect of intracellular Ang II) is, in part, mediated by PDGF production, secretion, and autocrine/paracrine cellular stimulation.

**Antisense Studies**

Our studies, using RT-PCR, show that naive H4-II-E-C3 cells possess ACE and renin mRNAs (Figure 7A) and that a rat...
renin antisense phosphorothioate oligomer eliminates the mitogenic effect of the Ang(–S)Exp product (Figure 8A). A corresponding scrambled-sequence control oligomer has no effect on cellular growth rate. The renin mRNA was further identified as renin 1A25 using primers specific for the alternatively spliced exon 1A (Figure 7B). After treatment with the antisense oligomer, renin 1A mRNA is no longer detectable (Figure 7B).

Discussion

Extracellular Ang II interaction with the G protein-linked AT₁ plasma membrane receptor primarily stimulates inositol phosphate and protein kinase C signal transduction26 but may also be coupled to Ras, Raf-1, MAP kinase, phospholipase A, Jak-Stat, and jun kinase pathways.14,27–29 Although Ang II generated through the renin-angiotensin system is commonly considered a classical endocrine hormone, Ang II can clearly be generated locally and act in an autocrine/paracrine or intracrine fashion. Indeed, a large body of evidence from many laboratories including our own (see Introduction) indicates that Ang II nuclear receptors exist and that Ang II has direct and specific nuclear effects. A number of hormones and growth factors have been found to have biologically significant intracellular activities.30–37 Whether nuclear Ang II must be internalized or whether it can be generated within (genetically unaltered) cells, remains to be determined. If Ang II can be generated intracellularly, the permissible cell types and conditions must yet be determined. Clearly, where growth factors have dual extracellular and nuclear roles, they may be secreted locally, internalized, and translocated to the nucleus, or directly transferred to the nucleus. Hepatoma-derived growth factor (HDGF), a heparin-binding growth factor similar to the fibroblast growth factors,38 seems to function through the latter mechanism. It is a secreted growth factor ubiquitously expressed and mitogenic for fibroblasts and endothelial cells. However, recent studies show that
HDGF is present in nuclei (its sequence contains a putative nuclear localization element) of cultured and fetal smooth muscle cells (SMCs) but not adult SMCs, suggesting that it is involved in proliferation during development and disease. Consistent with this hypothesis, HDGF is also present in adult SMC nuclei after rat aortic banding, and studies suggest that HDGF directly translocates to the nucleus rather than being locally secreted, internalized, and subsequently translocated to the nucleus. The authors suggest that HDGF can reside in the cytoplasm or nucleus or be secreted, depending on the cell type and function and cell cycle phase. Consistent with evidence that levels of intracellular HDGF may directly reflect the disease state of a cell, intracellular Ang II in myocardi um (myocytes and endothelial cells) of diabetic hypertensive patients may similarly reflect disease severity. A recent study suggests that intracellular Ang II levels in situ in hearts of diabetic patients are increased 3.4- and 3.1-fold, respectively, in myocytes and endothelial cells over levels present in nondiabetic patients. Intracellular Ang II levels appear to be increased an additional 2-fold in diabetic hypertensive patients compared with diabetic nonhypertensive patients. The authors suggest that local elevations of Ang II which accompany diabetes and hypertensive diabetes, in turn, enhance oxidative damage and activate cardiac cell apoptosis and necrosis. Elevated intracellular Ang II may, therefore, be both a marker of disease and contribute to disease progression.

The model system that we have constructed in the present study suggests that Ang(−S)Exp (Aogen lacking the N-terminal signal sequence required for secretion) is growth stimulatory for rat hepatoma cells. We chose to use H4-II-E-C3 cells because liver is a primary production site for plasma Aogen and because we had previously demonstrated that these cells possess renin and ACE mRNAs and, therefore, the potential to process Aogen to Ang II. H4-II-E-C3 cells when either transiently transfected or stably genetically altered with this plasmid show increased growth (at a 33% average increase in BrdU-labeled cells for stably transfected clones). The Ang(−S)Exp growth stimulatory effect is losartan-inhibited suggesting that it is conferred by Ang II; however, the growth effect is not blocked by candesartan (despite the fact that candesartan inhibits exogenous Ang II stimulation of these cells), or by losartan after cellular preincubation with candesartan. This suggests that the mitogenic effect of Ang(−S)Exp is not caused by extracellular Ang II accumulation.

We suggest that, in our studies, losartan, after AT1 receptor binding and internalization, blocks intracellular Ang II action. Conchon et al. have shown that about 17% of bound 1H-losartan is internalized into CHO cells genetically modified to express AT1 and AT2 receptors. This value is certainly significant but could be even higher for different cell types, depending on cell surface receptor expression levels. In addition, it has been proposed that losartan is sufficiently hydrophobic that it may freely penetrate cell membranes, a property that could be a function of cell membrane composition. Candesartan, on the other hand, appears to bind tightly to AT1 but to remain at the cell surface. Consistent with our hypothesis that losartan inhibits the Ang(−S)Exp growth stimulatory effect after cellular internalization via the AT1 receptor, PAO, which blocks receptor internalization, abolishes the losartan growth inhibitory effect in a DTT-reversible fashion. (DTT has been shown in other studies to inhibit PAO activity, presumably by reducing a disulfide bond required for PAO binding or PAO inhibition of receptor internalization.) In further support of our hypothesis, anti-Ang II antibodies, which we show to inhibit Ang II–stimulated growth of naive H4-II-E-C3 cells (by exogenously added Ang II), have no effect on growth of Ang(−S)Exp/pSVL transgenic cells; Ang II produced from Ang(−S)Exp appears to be produced and to remain within the cells (where it is inaccessible to antibodies). In contrast, anti-PDGF antibodies partially inhibit the Ang(−S)Exp growth stimulatory effect, suggesting that it is mediated, at least in part, through Ang II stimulation of downstream PDGF production. Consistent with this observation, steady-state PDGF mRNA levels are elevated an average of 2.2-fold in Ang(−S)Exp/pSVL compared with Ang(−S)Cntr/pSVL stably transfected cell lines. This observation, in turn, is consistent with earlier studies that demonstrate elevated transcription rates of several genes including PDGF (80% increase) after treatment of isolated liver nuclei with Ang II. Therefore, intracellular Ang II production appears to elevate extracellular PDGF levels (among other growth factors) and, thus, lead to autocrine/paracrine stimulation of cellular growth.

Intracellular renin production is also required for the mitogenic effect of Ang(−S)Exp; antisense oligomers complementary to renin mRNA are growth inhibitory for H4-II-E-C3 cells that are stably transfected with Ang(−S)Exp/pSVL. Furthermore, the renin mRNA transcribed corresponds to a recently identified alternative transcript.
resulting from an alternative transcription start site in intron 1 and an alternative splicing process.\textsuperscript{25} Translation of this mRNA is believed to result in a truncated prorenin, lacking a signal sequence and representing a cytosolic form of active renin. Our data suggest, therefore, that Ang II from Ang\textemdash(\textminus)S\textsubscript{Exp} is generated through a renin-dependent intracellular pathway.

Although the preponderance of available evidence suggests that the effects of intracellular angiotensin that we observe in Ang\textemdash(\textminus)S\textsubscript{Exp}\textsuperscript{3} transfected cells results from product binding at nuclear sites, angiotensin receptors have been identified associated with other intracellular organelles, and it is possible that such binding accounts for the phenomenon we have observed.\textsuperscript{4} Although less likely than a nuclear site of action, this is nonetheless consistent with intracrine angiotensin action.

In this model, we have identified a renin-dependent AT\textsubscript{1}-mediated effect which is blocked by losartan but not by candesartan. These studies of cells transgenic for constructs encoding angiotensinogen proteins devoid of secretory signals supports the hypothesis that angiotensin and a growing list of additional peptides, growth factors, and cytokines can generate biologically relevant effects by acting at an intracellular site. We believe that Ang II may act at the cell surface or within the nucleus and that it may be internalized or generated internally depending on cell type, cell age, genetic background, and cellular environment (or culture conditions). Because molecules smaller than 40 to 45 kDa generally diffuse freely between the cytoplasm and nucleus,\textsuperscript{37} internalized or intracellularly generated Ang II likely requires no signal for nuclear localization.

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References


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