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A Competitive Deletion Mutant Quantitative PCR Assay for Angiotensin-converting Enzyme mRNA in Smooth Muscle Cells

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To quantify angiotensin-converting enzyme (ACE) mRNA, we have developed a reverse transcription (RT)-coupled competitive PCR (RT–PCR) assay with a deletion mutant internal standard. The RT–PCR detects ACE mRNA from both human and bovine sources. ACE mRNA was detected in total RNA from cultured human saphenous vein smooth muscle cells (HuSV-SMCs) and from bovine pulmonary artery (BPA) SMCs. BPA-SMC expressed ninefold less ACE mRNA than BPA endothelial cells, and threefold less than HuSV-SMCs. Apparent amounts of ACE mRNA were 118,350 ±2,300 copies in HuSV-SMCs and 42,200 ±11,300 copies in BPA-SMCs per microgram of total cell RNA. The accuracy of the absolute values is subject to the limitations of the assumptions used to calculate them. These data support the hypothesis that components of the renin–angiotensin system are transcribed by SMCs.

A comprehensive assessment of eukaryotic gene transcription requires, in part, quantification of cellular mRNA levels. However, constitutive transcription from many genes does not provide sufficient amounts of mRNA for rigorous analysis by conventional methodologies like Northern blotting and RNase protection. Since the invention of PCR, an emerging trend has been to reverse-transcribe mRNA to cDNA with subsequent amplification of the cDNA by PCR to provide sufficient material for analysis.[1] This coupled RT–PCR has been refined to enable both relative and absolute quantification of mRNA.[1–3] Here, we describe a competitive RT–PCR assay for angiotensin-converting enzyme (ACE) using a deletion mutant internal standard for absolute quantification of ACE mRNA and use this assay to assess ACE gene transcription in smooth muscle cells (SMCs).

Conversion of angiotensin-1 to angiotensin-2 and inactivation of bradykinin are among the physiologically important ACE functions that complement the diverse ACE activity toward several other polypeptide substrates.[4,5] Endothelial cells from several species, including human and bovine, express ACE activity both constitutively and in a regulated manner on the plasma membrane as an ectoenzyme with the catalytic site readily available to blood-borne substrates. In addition, catalytically active ACE is shed into the circulation upon proteolysis of its membrane anchor. Thus, ACE is an important component of both local tissue and circulating renin–angiotensin systems. Using RT–PCR we now report that human saphenous vein (HuSV) and bovine pulmonary artery (BPA) SMCs have 118,350 ±2,300 copies and 42,200 ±11,300 copies, respectively, of ACE mRNA per microgram of total cellular RNA. These data extend ACE expression beyond endothelial and epithelial cells to include SMCs among the cells that transcribe the ACE gene.

MATERIALS AND METHODS

Cell Culture

BPA endothelial cells (BPA-ECs) were isolated from main stem arteries by enzyme isolation and cultured in RPMI-1640 medium containing 5% fetal bovine serum (FBS) (Sigma Chemical Co., St Louis, MO), 100 U/ml of penicillin (Marsam Pharmaceuticals, Cherry Hill, NJ), 100 μg/ml of streptomycin (Eli Lilly, Indianapolis, IN), and 1.25 μg/ml of amphotericin-B (GIBCO BRL, Gaithersburg, MD) as described previously.[6]

A modification of the method of Ross[7] was used for the isolation and culture of BPA-SMCs, and they were cultured under the same conditions described for BPA-ECs.

HuSV-SMCs were obtained from the Molecular Cardiology Research Laboratory of the New England Medical Center and cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS, L-glutamine, penicillin, streptomycin, and amphotericin B. Third to sixth passaged cells were used at a seeding density of 320,000/100-mm dish. Cells were maintained in culture at 37°C in a humidified chamber containing 5% CO_2, and media were replaced every 48 hr.

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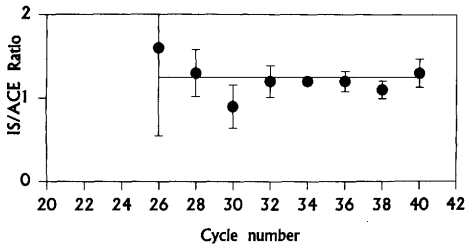

FIGURE 1 IS/ACE ratio vs. cycle number. Approximately equal amounts of BPA-EC cDNA and IS were subjected to 40 PCR cycles, and samples were removed after every other cycle from cycles 22–40. Samples were electrophoresed on 3% NuSieve/1% SeaKem agarose in TAE buffer for 90 min at 80 V and stained with ethidium bromide. ACE and IS products were determined by densitometry, and the ratio was plotted as a function of cycle number.

Endothelial cells were identified by their cobblestone appearance and the presence of factor VIII as determined by antibody staining. Smooth muscle cells contained muscle specific actin and were elongated in shape. By these criteria, there was no detectable cross contamination in the populations of cells studied.

Reverse Transcription

Total cell RNA was obtained from 3- to 4-day postconfluent cultured bovine and human cells with RNAzol B according to the manufacturer's instructions. Concentration was determined spectrophotometrically at 260 nm. RNA was serially diluted with diethyl pyrocarbonate-treated water containing 1 U/μl of RNase inhibitor and 3 mM dithiothreitol. RNA was primed with 660 pmoles of oligo$(dT)_{12-18}$ and reverse transcribed by Moloney murine leukemia virus reverse transcriptase (M-MuLV–RT), in a total volume of 30 μl containing 460 μM of each dNTP for 1 hr at 37°C as described previously.[8] After reverse transcription, samples were heated at 95°C for 5 min to denature the M-MuLV–RT and stored at −40°C until amplified by PCR.

Deletion Mutant Preparation

The deletion mutant internal standard (IS) was prepared from a PCR fragment generated by using primers 5′-GACTTTGACCCAGGGGCCAAGTTC-3′ (forward) and 5′-GTGGAGGCTGCGGTGGCGGATGCT-3′ (reverse) to amplify a 540-bp region of human ACE cDNA spanning nucleotides 3350–3889. This fragment was restricted with *Hae*II (New England Biolabs, Beverly, MA) at two sites 83 bp apart to yield three fragments of 171, 83, and 286 bp, which were resolved by electrophoresis on agarose. The 171- and 286-bp fragments were excised and ligated by overlap extension PCR to yield a 457-bp mutant of the original fragment with an 83-bp deletion that was purified on Geneclean (Bio101, La Jolla, CA) and used as the IS.

PCR

A master PCR reagent mixture was prepared such that each 50 μl contained 0.4 μMm each primer, 52 μM dNTPs, and 0.1 μl of digoxigenin (Dg) labeling mixture (consisting of 890 μM each of Dg-11-dUTP, dATP, dGTP, and dCTP) in 15 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% gelatin (pH 8.5). The mixture was designed to give a final 3% Dg-11-dUTP/dTTP ratio in the 50-μl PCR samples. A constant amount of cDNA target was added to the master mixture of PCR primers and buffer, and 50-μl aliquots were placed in several tubes. The deletion mutant IS was serially diluted (either 2- or 10-fold) and 9–9000 copies of IS were added to each 50 μl of PCR reagent mix. Samples were overlaid with light mineral oil and held at 80°C for hot start PCR. *Taq* polymerase (1.25 units) in 2 μl was added to each sample by underlaying, and PCR was carried out for 35–40 cycles for 30 sec at 94°C for denaturation, 1 min at 60°C for annealing, and 1 min at 72°C for extension. The last cycle extension was for 10 min. Primers 5′-TTACGTCAGGTACTTCGTCAGCTT - 3′ (forward) and 5′-CAGTCCACCAGCGGCTTGAAGTAG-3′ (reverse) amplified either a 249-bp region of human ACE cDNA between nucleotides 3394 and 3642 or the homologous region of bovine cDNA between nucleotides 3372 and 3620. Concomitantly, a 166-bp fragment of the IS was amplified. Thus, the RT–PCR detects ACE mRNA from both human and bovine sources. Primer pairs span several introns such that genomic DNA is not amplified.

Quantification

After PCR, 10-μl sample aliquots were electrophoresed on 3% NuSieve/1% SeaKem agarose in Tris/acetic acid/EDTA (TAE) buffer for 90 min at 80 V, stained with ethidium bromide, and photographed. Alternately, when greater sensitivity was required, fragments were transferred to uncharged nylon and UV cross-linked as described previously.[8] The membrane was treated sequentially, for 30 min each time, with a blocking reagent that consisted of 5% HiPure liquid gelatin (Norland, New Brunswick,

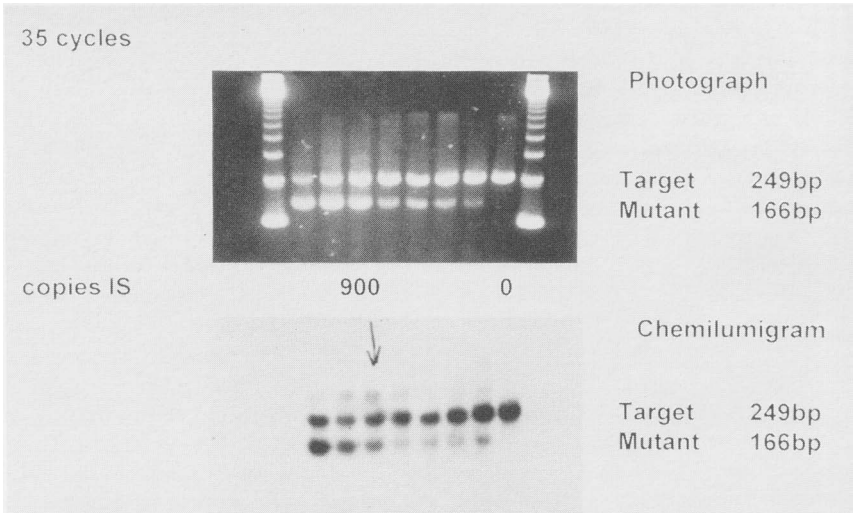

FIGURE 2 Separation and detection of PCR products. PCR was for 35 cycles with BPA-EC cDNA and 0, 90, 180, 360, 450, 900, 1800, and 3600 IS copies. Agarose electrophoresis was performed as described in Fig 1. (*Top*) Photograph of ethidium bromide-stained gel; (*bottom*) chemilumigraph of Dg-labeled products. The arrow indicates a visual approximation of the estimated equivalence point. The actual equivalence point was determined from the data in Fig. 3A. Right and left end lanes contain a 123-bp DNA calibration ladder.

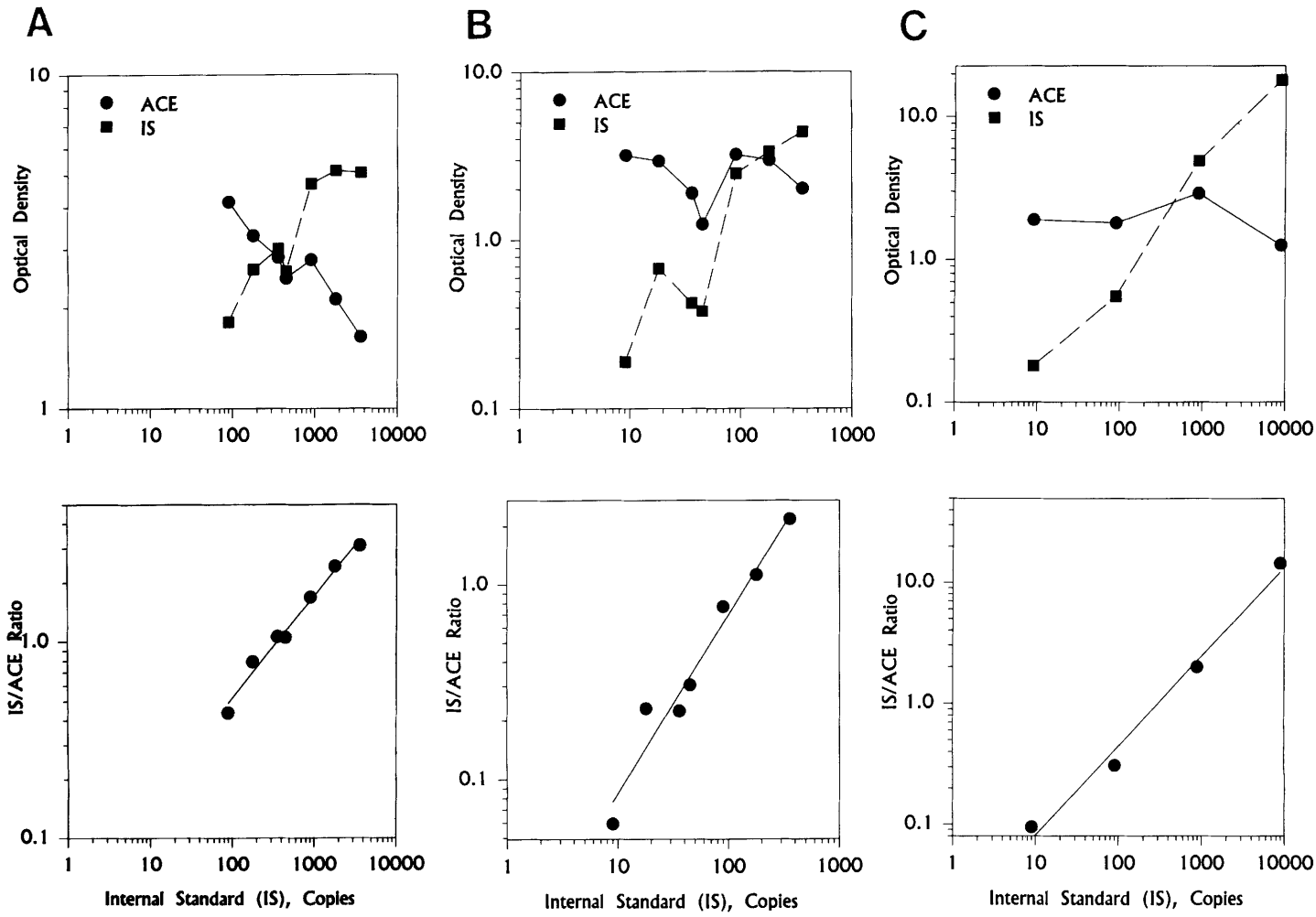

FIGURE 3 Competitive quantitative PCR with EC and SMC cDNAs. PCR, electrophoresis, and densitometry were performed as described in Materials and methods. (*Top* panels) ACE and IS optical densities from photographs of ethidium bromide-stained gels were plotted vs. copies of IS in each sample. (*Bottom* panels) After correcting for molecular weight differences, IS/ACE ratios were calculated from *top* panel data and plotted vs. IS copies. Equivalence points, where copies of ACE cDNA and IS are equal, were determined from the abscissa at the point on each line where the ordinate value is 1. (*A*) BPA-ECs (4 ng of RNA per tube); (*B*) BPA-SMCs (12 ng of RNA per tube); (*C*) HuSV-SMCs (15 ng RNA per tube).

NJ), 0.1% Tween 20, 150 mM sodium phosphate, 140 mM sodium chloride, and 0.02% sodium azide (pH 7.4), followed by anti-Dg Fab fragment conjugated to alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) diluted 1:10,000 in blocking reagent. Subsequent washing, detection with the chemiluminescent substrate AMPPD (Tropix, Bedford, MA), and film exposure were done as described previously.[8] Densitometry was done with a Millipore densitometer with Visage v4.6p software (Millipore, Bedford, MA). Because the target is larger than the mutant, target optical density was multiplied by a correction factor of 0.67 to normalize the value before calculating the molar ratio of a 166-bp mutant to a 249-bp target.

Optical densities for ACE and IS were plotted versus copies of IS to assess amplification as a function of IS input. The equivalence point, where target and IS copies are equal, was determined from a plot of IS/ACE ratio versus copies of IS. This value was divided by 0.4 to compensate for the <100% reverse transcription efficiency that was presumed to be 40% as determined from the manufacturer's literature and elsewhere.[9] Also, the value was multiplied by 2 to compensate for differential amplification during the first PCR cycle where the single-stranded cDNA target was rendered double stranded while the double-stranded IS was amplified geometrically. Corrected values are reported as copies of ACE mRNA per microgram of total cellular RNA.

RESULTS AND DISCUSSION

Southern blotting with a Dg-labeled human ACE probe[8,10] confirmed that the primers amplified the correct targets (data not shown). Additional confirmation was obtained by restricting with *Ava*II, *Bcl*I, *Hae*II, and *Nci*I both the target from which the mutant was prepared and a 360-bp nested PCR product amplified from the deletion mutant. Restricted fragments were electrophoresed on 3% NuSieve/1% SeaKem agarose in TAE buffer and stained with ethidium bromide (data not shown). Both *Hae*II sites in the target that spawned the mutant and the single restored *Hae*II site in the mutant were cut correctly. Also the single unperturbed *Ava*II and *Nci*I sites in both the target and mutant were cut cor-

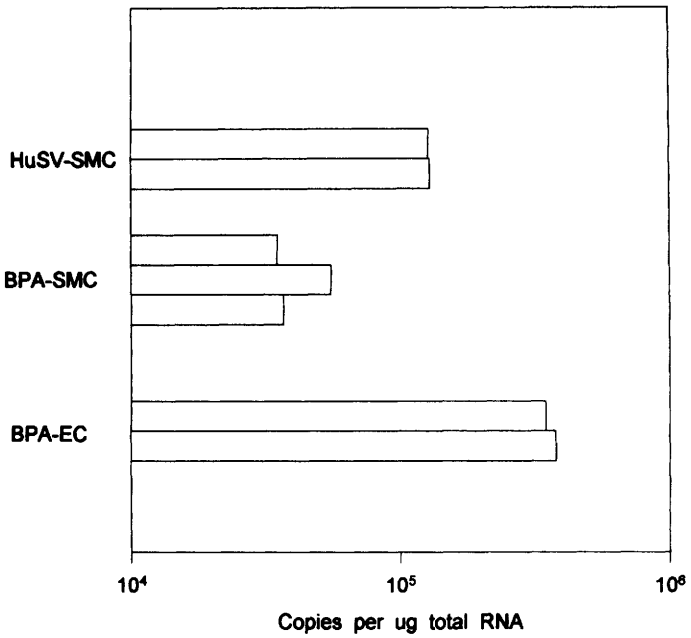

FIGURE 4 Copies of ACE mRNA per microgram of total cell RNA. Values for HuSV-SMCs, BPA-SMCs, and BPA-ECs, were corrected for reverse transcription efficiency and single-stranded cDNA as described in Materials and Methods. HuSV-SMC and BPA-EC data were from duplicate determinations; BPA-SMC data were from triplicate determinations.

rectly. A single *BclI* site in the target that was within the excised fragment removed upon preparing the mutant was no longer recognized in the mutant but was cut correctly in the target.

To ensure that reverse transcription occurred efficiently, 10 μg of total cell RNA was serially diluted in twofold decrements prior to reverse transcription. Subsequently, PCR was performed with these cDNA samples. PCR product was not proportional to RNA when 10 μg RNA was used for RT–PCR but did become proportional at lower RNA input of ~2 μg or less. Thus, PCR inhibitors in total RNA could be offset by titration of the RNA prior to reverse transcription. For these experiments, all RT–PCR was done with RNA concentrations that did not inhibit PCR.

To ensure that the IS/ACE ratio did not vary when PCR was extended through the exponential range and into the plateau region, the IS/ACE ratio was determined every two cycles for up to 40 cycles. The IS/ACE ratio remained constant and was independent of cycle number (Fig 1). Thus, with a homologous IS, competitive quantitative RT–PCR for ACE mRNA can be performed at any point within the entire range of the amplification efficiency curve. These data support a similar observation reported initially by Gilliland et al. for cytokine mRNA[3] and confirmed for other targets.[11–14]

PCR products were Dg-labeled routinely to aid with detection of samples that expressed low target mRNA levels and showed faint ethidium bromide-stained PCR fragments even after 40 cycles. For those samples, densitometric analysis was performed from the chemilumigraph. In general, the chemilumigraph mimicked the pattern shown on the photograph (Fig. 2). However, in some cases, IS/ACE ratios obtained from the chemilumigraph differed from those obtained from the photograph because of uneven transfer. For samples where sufficient DNA was available for adequate ethidium bromide staining, the gel photograph was preferred for densitometry. Optical densities for IS and ACE were plotted, both separately and also as an IS/ACE ratio, as a function of IS copies added to the samples (Fig. 3).

The benchmark for ACE expression is the endothelial cell. Thus, we used BPA-ECs as a positive control for quantification of ACE gene expression by RT–PCR. For each sample, serial dilutions of IS were added to a constant amount of target cDNA and subjected to 35–40 PCR cycles. Experiments were repeated with RNA from HuSV–SMCs and BPA-SMCs. Because the IS used here is cDNA rather than cRNA, the RT–PCR quantitates cDNA equivalents rather than mRNA. From an analysis of published data,[9] we assumed that the efficiency of reverse transcription was, on average, 40%. We based our calculation of absolute mRNA levels on the validity of this assumption. However, we recognize that the actual absolute amount of ACE mRNA may be skewed if this assumption is invalid. Thus, absolute values should be taken as apparent values subject to the limitations of the assumptions used to calculate them. Nevertheless, relative ACE gene expression can be deduced without the need to estimate RT efficiency. From two experiments, we found 348,100 and 379,700 copies of ACE mRNA per microgram of BPA-EC total RNA (Fig. 4). Correspondingly, from three experiments, BPA–SMCs had 34,800, 55,300, and 36,700 copies of ACE mRNA per microgram of total RNA. Thus, on a relative basis, bovine ECs expressed ninefold more ACE mRNA than SMCs per microgram of total RNA. HuSV-SMCs had 116,700 and 120,000 copies of ACE mRNA per microgram total RNA, approximately threefold the BPA-SMC amounts. These data complement our qualitative analysis of ACE gene transcription by human pulmonary artery SMC.[15]

Local renin–angiotensin systems (RAS) play key roles in the physiological homeostasis of heart,[16] brain,[17] and kidney.[18] Whether or not complete RAS, including angiotensinogen, renin, ACE, angiotensin-2, and angiotensin-2 receptors, exist in tissues or cells constituting paracrine and autocrine systems, respectively, remains equivocal. SMC have been reported to have angiotensinogen,[19] renin activity,[20] ACE activity,[21] and angiotensin-2 receptors.[22–24] However, it is not clear whether all of these components are transcribed and translated by the SMCs or are taken up from the extracellular milieu. Thus, unequivocal evidence for ACE gene transcription by SMCs is a prerequisite for SMC autocrine RAS function. The RAS end product, angiotensin-2, has growth-promoting activity in general[25] and is mitogenic for SMCs.[26,27] An SMC autocrine RAS has the potential to contribute pathologically to the vascular remodeling sequelae of hypertension,[28] to myocardial hypertrophy,[29] and to restenosis after angioplasty. The data presented here demon-

strate that HuSV-SMCs and BPA-SMCs transcribe the ACE gene. It is therefore highly probable that ACE catalytic activity in SMCs derives from translation of the ACE message and not as a result of extracellular ACE sequestration. Whether endogenous production of all other RAS components can be established in SMCs remains to be explored.

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REFERENCES

1. Chelly, J., J.-C. Kaplan, P. Maire, S. Gautron, and A. Kahn. 1988. Transcription of the dystrophin gene in human muscle and non-muscle tissues. *Nature* **333:** 858–860.
2. Wang, A.M., M.V. Doyle, and D.F. Mark. 1989. Quantitation of mRNA by the polymerase chain reaction. *Proc. Nat. Acad. Sci.* **86:** 9717–9721.
3. Gilliland, G., S. Perrin, K. Blanchard, and H.F. Bunn. 1990. Analysis of cytokine mRNA and DNA: Detection and quantitation by competitive polymerase chain reaction. *Proc. Natl. Acad. Sci.* **87:** 2725–2729.
4. Skidgel, R.A. and E.G. Erdos. 1987. The broad substrate specificity of human angiotensin 1 converting enzyme. *Clin. Exp. Hypertens. Part A Theory Pract.* **A9**: 243–259.
5. Ehlers, M.R.W. and J.F. Riordan. 1989. Angiotensin-converting enzyme: New concepts concerning its biological role. *Biochemistry* **28:** 5311–5318.
6. Lee, S.L. and B.L. Fanburg. 1986. Serotonin uptake by bovine pulmonary artery endothelial cells in culture. I. characterization. *Am. J. Physiol.* (*Cell Physiol.* **19**) **250:** C761–C765.
7. Ross, R. 1971. The smooth muscle cell. II. Growth of smooth muscle in culture and formation of elastic fibers. *J. Cell Biol.* **50:** 172–186.
8. Lanzillo, J.J. 1991. Chemiluminescent nucleic acid detection with digoxigenin-labeled probes: A model system with probes for angiotensin converting enzyme which detect less than one attomole of target DNA. *Anal. Biochem.* **194:** 45–53.
9. Bouaboula, M., P. Legoux, B. Pessegue, B. Delpech, X. Dumont, M. Piechaczyk, P. Casellas, and D. Shire. 1992. Standardization of messenger RNA titration using a polymerase chain reaction method involving coamplification with a multispecific internal control. *J. Biol. Chem.* **267:** 21830–21838.
10. Lanzillo, J.J. 1990. Preparation of digoxigenin-labeled probes by the polymerase chain reaction. *BioTechniques* **8:** 621–622.
11. Pannetier, C., S. Delassus, S. Darche, C. Saucier, and P. Kourilsky. 1993. Quantitative titration of nucleic acids by enzymatic amplification reactions run to saturation. *Nucleic Acids. Res.* **21:** 577–583.
12. Diviacco, S., P. Norio, L. Zentilin, S. Menzo, M. Clementi, G. Biamonti, S. Riva, A. Falaschi, and M. Giacca. 1992. A novel procedure for quantitative polymerase chain reaction by coamplification of competitive templates. *Gene* **122:** 313–320.
13. Nedelman, J., P. Heagerty, and C. Lawrence. 1992. Quantitative PCR with internal controls. *Comput. Appl. Biosci.* **8:** 65–70.
14. Santagati, S., E. Bettini, M. Asdente, M. Muramatsu, and A. Maggi. 1993. Theoretical considerations for the application of competitive polymerase chain reaction to the quantitation of a low abundance messenger RNA: Estrogen receptor. *Biochem. Pharmacol.* **46:** 1797–1803.
15. Aschoff, J.M., D. Lazarus, B.L. Fanburg, and J.J. Lanzillo. 1994. Relative quantification of angiotensin-converting enzyme mRNA in human smooth muscle cells, monocytes, and lymphocytes by the polymerase chain reaction. *Anal. Biochem.* **219:** 218–223.
16. Lindpainter, K., and D. Ganten. 1991. The cardiac renin-angiotensin system: A synopsis of current experimental and clinical data. *Acta Cardiol.* **46:** 385–397.
17. Bunnemann, B., K. Fuxe, and D. Ganten. 1993. The renin-angiotensin system in the brain: An update 1993. *Regul. Pept.* **46:** 487–509.
18. Schunkert, H., J.R. Ingelfinger, and V.J. Dzau. 1991. Evolving concepts of the intrarenal renin-angiotensin system in health and disease: Contributions of molecular biology. *Renal Physiol. Biochem.* **14:** 146–154.
19. Dzau, V.J. 1988. Circulating versus local renin-angiotensin system in cardiovascular homeostasis. *Circulation* **77:** I-4–I-13.
20. Re, R.N., J.T. Fallon, V.J. Dzau, S. Quay, and E. Haber. 1982. Renin synthesis by canine aortic smooth muscle cells in culture. *Life Sci.* **30:** 99–106.
21. Andre, P., C. Schott, H. Nehlig, and J.C. Stoclet. 1990. Aortic smooth muscle cells are able to convert angiotensin-I to angiotensin-II. *Biochem. Biophys. Res. Commun.* **173:** 1137–1142.
22. Roberts, T.B., S.T. Gaa, and J.S. Allen. 1986. Identification and characterization of functional angiotensin II receptors on cultured heart myocytes. *J. Pharmacol. Exp. Ther.* **236:** 438–444.
23. Griendling, K.K., S.E. Rittenhouse, T.A. Brock, L.S. Ekstein, M.A. Gimbrone Jr., and R.W. Alexander. 1986. Sustained diaclyglycerol formation from inositol phospholipids in angiotensin II-stimulated vascular smooth muscle cells. *J. Biol. Chem.* **261:** 5901–5906.
24. Griendling, K.K., T. Tsuda, B.C. Berk, and R.W. Alexander. 1989. Angiotensin II stimulation of vascular smooth muscle. *J. Cardiovasc. Pharmacol.* **14:** s27–s33.
25. Schelling, P., D. Ganten, G. Speck, and H. Fischer. 1979. Effects of angiotensin II and angiotensin II antagonist saralasin on cell growth and renin in 3T3 and SV3T3 cells. *J. Cell. Physiol.* **98:** 503–513.
26. Campbell-Boswell, M. and A.L. Robertson. 1981. Effects of angiotensin II and vasopressin on human smooth muscle cells in vitro. *Exp. Mol. Pathol.* **35:** 265–276.
27. Geisterfer, A.A.T. and G.K. Owens. 1986. Hypertrophic response of cultured vascular smooth muscle cells to angiotensin II. *Fed. Proc.* **45:** 584.
28. Re, R.N. 1989. The cellular biology of angiotensin: Autocrine and intracrine actions in cardiovascular tissues. *J. Mol. Cell. Cardiol.* **21:** 63–69.
29. Dzau, V.J. 1993. Tissue renin-angiotensin system in myocardial hypertrophy and failure. *Arch. Intern. Med.* **153:** 937–942.

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