0026-895X/97/020201-08$3.00/0

MOLECULAR PHARMACOLOGY, **51:**201–208 (1997).

β-Adrenoceptor Activation-Induced Placental Prorenin Secretion Is Mediated by Increased Renin Messenger RNA and Protein Synthesis

GREGORY J. DOWNING, BINGFANG YAN, and ALAN M. POISNER

Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, Kansas 66160

Received July 9, 1996; Accepted October 21, 1996

SUMMARY

Activation of β-adrenoceptors has been shown to promote renin secretion in both human kidney and placenta. In kidney, the enhanced secretion is immediately observed, and mobilization of renin in the storage granules accounts for such a rapid response. In contrast, the enhanced secretion in placenta is delayed for 6–12 hr after receptor activation and consists almost entirely of the renin precursor prorenin. It is hypothesized that newly synthesized rather than stored enzyme is responsible for the enhanced secretion in human placenta. To test this hypothesis, placental explants were cultured in the presence or absence of the protein synthesis inhibitor cycloheximide, and prorenin concentrations in the tissue and medium were measured. Dobutamine and terbutaline, β_1- and β_2-adrenoceptor agonists, evoked 17- and 5-fold increases in secretion, respectively. Tissue content of prorenin in response to the treatment was increased by a similar magnitude, yet values were consistently $<$10% of medium concentrations. The increases in prorenin concentrations in both medium and tissue, however, were markedly attenuated by cycloheximide, suggesting that prorenin synthesis in response to β-adrenoceptor activation is required. Reverse transcription coupled with polymerase chain reaction revealed that renin mRNA levels were increased by 3–8-fold and occurred before increases in tissue and medium prorenin, indicating that increased renin mRNA levels are responsible for the increased synthesis of prorenin. Explants cultured in the presence of actinomycin D, an inhibitor of transcription, did not show the agonist-induced prorenin mRNA levels or enhancement of its secretion. The peak levels of renin mRNA were reached after 6 hr of incubation, were sustained at similar levels after 24 hr, and were not affected by cycloheximide. These findings are consistent with the notion that enhancement of renin mRNA and de novo protein synthesis are required for prorenin secretion induced by activation of placental β-adrenoceptors.

Components of the RAS have been localized in human placental tissues, yet their physiological importance in reproduction remains undetermined (1). Suggested roles for this extrarenal RAS include regulatory influences on placental circulation (2–4), secretion of placental hormones (5), and angiogenic effects on the early gestation vascular bed (6).

It has been demonstrated that activation of β-adrenoceptors promotes prorenin secretion from placental tissues by a cAMP-dependent mechanism (7). Characteristic of this response was a 6–12-hr period of incubation before the release of prorenin, which distinguishes the placental response from that of the kidney. Delayed induction brings into question the possibilities of the requirement of protein synthesis to mediate transcription activation or impairment in mRNA degradation.

During recent years, studies of renin gene transcription regulation in a number of placental cell preparations have indicated the importance of 5′-flanking DNA sequences (8–10). Of particular interest are the cAMP-induced mechanisms of renin gene expression involving pituitary-specific factor Pit-1 and CRE binding sites (11). In contrast to the kidney, it is generally accepted that in the placenta, enhanced prorenin secretion is largely accounted for by renin gene expression. The lack of identification of specific CREBs in placental cells and the possibility of alternative regulatory 5′-flanking regions for cAMP-mediated transcriptional control indicate that the mechanisms by which cAMP influences renin gene transcription are not completely understood. An alternative mechanism of cAMP-regulated gene expression has been offered; it was shown in a juxtaglomerular cell preparation that cAMP enhances the stability of renin mRNA (12). Also, the delay of prorenin secretion in response to β-adrenoceptor activation may be attributed in part to intracellular processing events that occur after renin gene transcription. In fact, recent evidence in a human pulmonary carcinoma cell line indicated the enhancing effects of cAMP on renin mRNA in a post-transcriptional manner (13).

ABBREVIATIONS: RAS, renin-angiotensin system; CRE, cAMP-response element; CREB, cAMP-response element-binding protein; mutREN, mutant renin; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription; SSC, standard saline citrate.

We therefore examined the effects of placental β-adrenoceptor activation on placental prorenin content, secretion, and renin mRNA levels and evaluated the effects of transcription and protein synthesis inhibitors on these responses.

Experimental Procedures

Placental explant culture. The study procedure was approved by the Institutional Review Committee at the University of Kansas Medical Center. The model used in this study was a modified version of the human placental explant system described previously (7, 14). Briefly, the explant was elevated on a wire mesh screen to allow complete submersion while maintaining proximity to the air/medium interface. Explants were incubated in CMRL 1060 medium containing 0.1% bovine serum albumin, 50 μg/ml gentamicin, and 25 μg/ml ampicillin. Unless stated otherwise, the medium was changed at 24-hr intervals. At the end of each incubation period, tissues were rinsed, blotted, placed in liquid nitrogen, and stored at −70°.

Renin assay. Total renin (active renin plus prorenin) was assayed after trypsin activation of the samples and radioimmunoassay of angiotensin I (^{125}I-angiotensin I; DuPont-New England Nuclear, Boston, MA) generated from sheep substrate (15). For the assay, prorenin was converted to active renin through exposure to bovine trypsin (2 μg/ml) at room temperature for 30 min. The reaction was terminated by the addition of soybean trypsin inhibitor (100 μg/ml). Active renin was also measured separately from each sample without the addition of trypsin. The enzymatic reaction was conducted in the presence of 5 mM EDTA, pH 8.0, and sheep renin substrate for 1 hr at 37°. Human renin (National Institute for Biological Standards, London, UK) was used as an internal standard. For each assay, a standard curve was generated [0.039–5 μunits/tube (Goldblatt units)] using samples diluted with culture medium to reach the linear portion of the curve. Each sample was assayed in duplicate. It is recognized that the vast majority of renin present in placental tissues is in the form of prorenin (16). Therefore, unless specified otherwise, values reported are with reference to prorenin concentrations and indicated as μunits of prorenin/mg of explant protein.

Protein assay. Each placental explant was suspended in 1 ml of 5 mM EDTA, pH 8.0, and sonicated twice for 15 sec followed by centrifugation at 3000 × *g* for 20 min. The supernatant fraction was collected for protein determination using bovine serum albumin as a standard (17).

RNA isolation. Total RNA was isolated from frozen placental tissues according to the procedure of Chomczinski and Sacchi (18). The amount of total RNA obtained varied from 2 to 4 μg/mg of placenta (wet weight). The integrity of the placental RNA was evaluated by agarose gel electrophoresis.

RT-PCR. The renin mRNA levels in placental explants treated with β-adrenoceptor agonists were determined by two methods in which the PCR technique was used. In the semiquantitative method, the amount of renin mRNA is compared with that of GAPDH, which is unaffected by β-adrenoceptor activation. Second, a quantitative measure of renin mRNA levels was performed by using a mutREN construct, which served as the template for the generation of cRNA. The resultant cRNA was quantified and used as a reference in the PCRs for the samples. Four micrograms of total RNA was used for RT-PCR. The RNA was denatured at 65° for 5 min in a reaction mixture containing 1 unit of RNasin, 0.5 mM dNTP, 10 pmol of oligo(dT) in 50 mM Tris·HCl, pH 8.3, 75 mM KCl, and 5 mM $MgCl_2$ in a final reaction volume of 19 μl. After the mixture was cooled at 4°, 1 unit of avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) was added, and the mixture was incubated at 42° for 45 min and then at 52° for 15 min.

The primers for the amplification of renin cDNA were 5′-GATGGATGGAGAAGGATG-3′ (forward; nucleotides 4–21) and 5′-AATCTCGCCATAGTACTG (reverse; nucleotides 253–270) corresponding to a sequence spanning intron A of the human renin precursor gene sequence (19). The PCRs were performed with 1 μl of cDNA using 2.5 units of *Taq* polymerase; 200 μM concentration each of dCTP, dTTP, dCTP, and dATP; 50 pmol of each oligonucleotide PCR primer; 10 mM Tris·HCl, pH 8.3; 50 mM KCl; and 2.5 mM $MgCl_2$ in a total volume of 100 μl. After the hot start (3 min at 94°; 80° hold), the samples were subjected to 25 cycles of 1 min at 94°/1 min at 56° followed by an extension step at 72° for 1 min with a final extension period of 10 min. For coamplification experiments, primers used for GAPDH cDNA were as follows: 5′-GCTTTTAACTCTGGTAAAGTGG-3′ (forward) and 5′-TCACGCCACAGTTTCCCGGAGG-3′ (reverse) corresponding to nucleotides 64–85 and 582–603 of the GAPDH coding sequence (20). These primers were designed from the 5′ region of each DNA sequence to produce a 267-bp product for renin cDNA and a 585-bp product for GAPDH. A 10-μl sample of the PCR mixture was applied to a 1% agarose gel and electrophoresed at 50 V for 1 hr.

Southern blot analysis. Each 10-μl sample was electrophoresed at 50 V for 1 hr in a 1% agarose gel with 1× TAE buffer (20 mM Tris acetate, 5 mM EDTA, pH 8.0). To transfer the PCR-amplified cDNA to a hybridization membrane, the DNA was denatured by soaking the gel in 500 ml of 0.5 M NaOH and 1.5 M NaCl for 30 min and then neutralized by soaking in 500 ml of Tris·HCl 0.5 and 1.5 M NaCl, pH 8.0, for 1 hr. The DNA was transferred to a hybridization membrane (Quantum Yield, Promega) that was presoaked in 10× SSC (1× = .15 M NaCl, .015 M sodium citrate) for 15 min using a vacuum-blotting system (Pharmacia LTB, Piscataway, NJ) at 50 cm of H_2O for 2 hr, after which the membrane was removed and soaked in 2× SSC for 15 min. The membrane was air dried and then baked for 2 hr at 80°, and the DNA was cross-linked to the membrane by exposure to 1200 μJ of UV light for 1 min. The hybridization was conducted using alkaline phosphatase-conjugated oligonucleotide probes with chemiluminescence detection (21, 22). Each step was performed in a rotating hybridization oven at 42–45°. The membrane was placed in a hybridization canister with 50 ml of a commercial blocking solution for 2–4 hr. After incubation for 5 min, 90 fmol of each hybridization probe (renin: 5′-TTCCTCAAGAGAATGCCCTCAATCCGAGAAAGCCTGAAGG-3′; GAPDH: 5′-TGCTGGCGCTGACTACGTCGTGGAGTCCACTGGCGTCT-3′) was added to the solution and incubated for an additional 30 min. The probe solution was removed, and the membrane was washed twice with 50 ml of 1% sodium dodecyl sulfate and 1× SSC for 5 min. The membrane was then washed with 50 ml of 100 mM diethanolamine and 1 mM $MgCl_2$, pH 10.0, for 5 min and incubated with 5 ml of chemiluminescent substrate 2-*o*-spiroadamantane-4-methoxy-4-[3–2′-phosphoryloxy]phenyl-1,2-dioxetone (Tropix, Houston, TX). An autoradiogram of the membrane was then exposed to radiographic film (Eastman Kodak, Rochester, NY). Individual bands from the autoradiographs representing renin and GAPDH cDNA were compared by densitometric assessment. Each lane from the autoradiograph was scanned with a densitometer (CS-9000; Shimadzu, Kyoto, Japan). Peak measurements for renin cDNA were expressed in relationship to GAPDH cDNA measurements.

Construction of the internal control and synthesis of internal control RNA. A plasmid was prepared for the generation of internal control RNA by inserting a 103-bp fragment of human GAPDH cDNA into a portion of the renin gene. The GAPDH fragment was generated by PCR, using a forward primer encoding for an *Xho*II restriction site (5′-GAGCGAGATCCCTCCAAAATCAAGTGGG-3′, nucleotides 235–262) and a reverse primer with an additional *Xho*II restriction site (5′-CCTTTTGGATCCGCCCTGCAAATGAGGC-3′, nucleotides 332–354). This PCR fragment was cleaved with *Xho*II and introduced into pGEM-4Z (Promega) containing renin cDNA (nucleotides 1–310). The subsequent construct was digested with *Eco*RI and *Sma*I, yielding a 370-bp fragment and then subcloned into pBluescript vector (Stratagene, San Diego, CA) containing an oligo-d(A) tail inserted at the *Hin*dIII site. After digestion with *Sal*I, the mutREN served as a template for *in vitro* transcription by T7 RNA polymerase to generate control RNA (Promega). The

resultant mutREN control RNA was quantified by absorbance at 260 nm, and the number of control RNA molecules was calculated using the molecular weight of the control RNA and Avogadro's number. Total RNA (1 μg) from placental explants was spiked with 2×10^7 molecules of mutREN control RNA, and the mixture was reversed transcribed as described above. Seven serial dilutions of 5 μl of the cDNA mixture were used for PCR. PCR was performed at a final concentration of 1× PCR buffer [2.5 mM $MgCl_2$, 100 μM concentration of dNTPs, 50 pmol of each the renin cDNA primers (forward, 5′-GATGGATGGAGAAGGATG-3′; reverse, 5′-AATCTCGCCATAGTACTG), 1×10^6 cpm of ^{32}P-end-labeled primer, and 2.5 units of *Taq* polymerase]. The DNAs were separated on a 4% NuSieve/agarose (3:1) gel and revealed 267- and 370-bp products for the wild-type renin and mutREN, respectively. Specific radioactive bands were quantified on a PhosphoImager (Molecular Dynamics, Sunnyvale, CA) after transfer of emitted radioactivity to a phosphor plate. Because the reaction rates of mutREN control RNA and placental mRNA are identical within the exponential phase of the PCR, this protocol permitted the construction of a standard curve for mutREN and extrapolation of renin mRNA molecules in placental samples. The data shown indicate the number of renin mRNA molecules present in 100 ng of total RNA. Four to six RNA isolations and RT-PCR assays were conducted for each control and treatment value.

Materials. Terbutaline, actinomycin D, cycloheximide, ampicillin, and gentamicin were purchased from Sigma Chemical (St. Louis, MO). Dobutamine HCl was a gift from Eli Lilly (Indianapolis, IN). Avian myeloblastosis virus reverse transcriptase, oligo(dT) primers, oligonucleotide labeling and detection system, and RNase inhibitor were purchased from Promega. *Taq* polymerase was purchased from Perkin-Elmer Cetus (Norwalk, CT).

Statistical analysis. Unless specified otherwise, data are presented as mean ± standard error. For concentration-response and time course experiments, a minimum of four placentas, with a minimum of six replicates from each, were used. Statistical analyses included paired Student's t test and an analysis of variance for repeated measures with Dunnett's multiple-comparison test to determine differences between groups. Differences were considered statistically significant when $p < 0.05$.

Results

Effects of terbutaline and dobutamine on prorenin secretion. To examine the effect of β-adrenoceptor activation on prorenin secretion, placental explants were incubated in the presence of dobutamine, a selective agonist of β_1-adrenoceptors, and terbutaline, a selective agonist for β_2-adrenoceptors. The concentration-response effect of these β-agonists on placental explant prorenin content and release was evaluated after 24 hr of incubation. Stimulation of prorenin release was observed with both β-agonists in a concentration-dependent fashion (Fig. 1, *top*). The maximal response to dobutamine exceeded that of terbutaline by >100%. The EC_{50} values for dobutamine and terbutaline were 10.8 ± 0.8 and 28.3 ± 1.3 μM, respectively. The corresponding tissue concentrations of prorenin were also increased in response to the β agonists and reflected the responses observed in the medium (Fig. 1, *bottom*). Maximal tissue prorenin concentrations in explants incubated with dobutamine and terbutaline were 17.1 ± 2.0 and 6.1 ± 0.4 μunits/mg of protein, respectively, representing ~10% of the values measured in the medium.

The stimulatory effects of β-adrenoceptor activation on placental prorenin were also studied as a function of incubation time (Fig. 2, *top*). Placental explants were incubated in

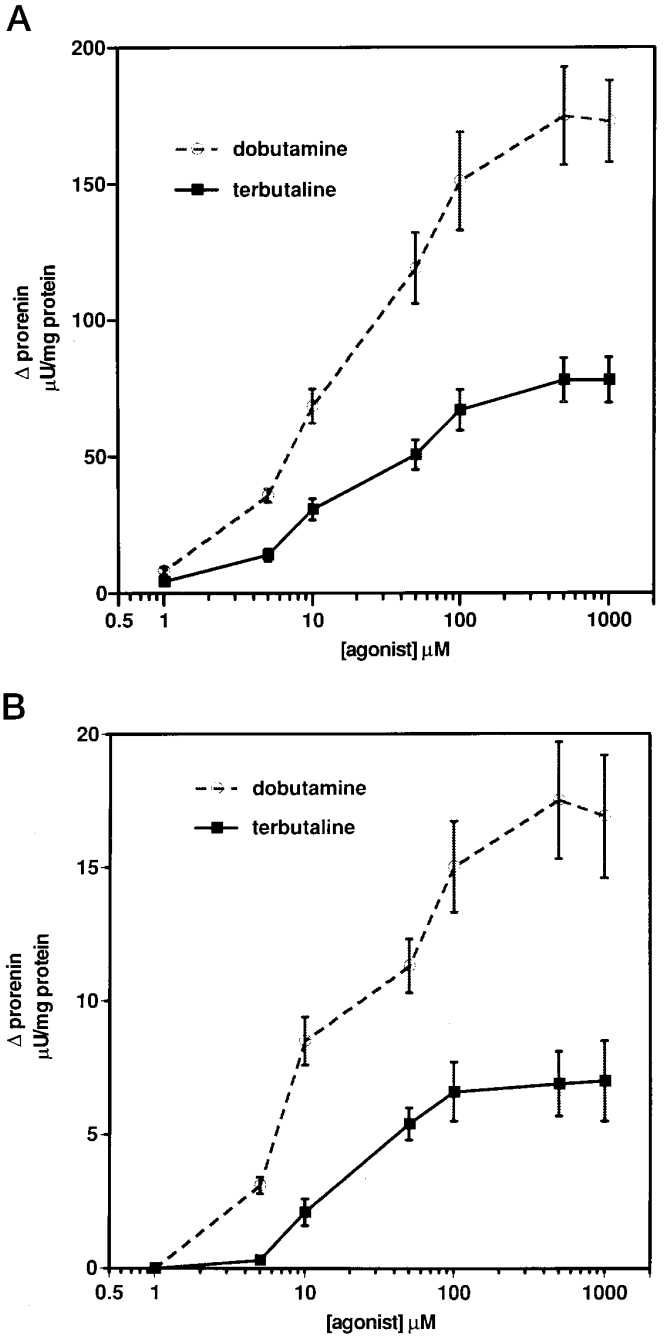

Fig. 1. Concentration-response effects of β-adrenoceptor activation on placental explant prorenin. *Top*, effects of dobutamine and terbutaline on medium concentrations of prorenin as determined after 24 hr of incubation. Media prorenin concentrations from control explants were 12.3 ± 1.8 μunits/mg of protein. *Bottom*, placental explant tissue concentrations of prorenin after 24 hr of incubation with dobutamine or terbutaline. The tissue concentration of prorenin in nonincubated tissue was 3.2 ± 0.3 μunits/mg of protein. Values represent mean ± standard error of six replicates from four individual placentas.

the presence or absence of β-adrenoceptor agonists (100 μM) with medium and tissue collected at various times. Prorenin concentrations in the medium of control explants reached 9.6 ± 0.6 μunits/mg of protein at 24 hr. Significant increases in medium prorenin concentrations were observed in explants incubated with β-adrenergic agonists only after 12 hr of incubation. The peak values for medium prorenin in re-

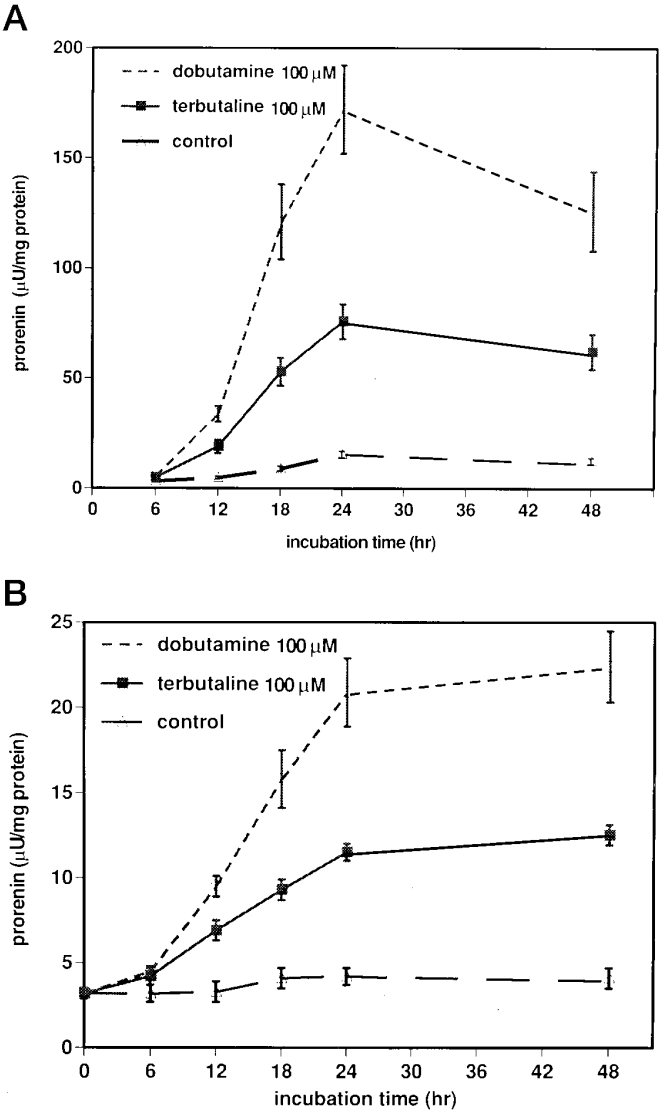

Fig. 2. Time course of effects of β-adrenoceptor activation on placental explant prorenin. *Top*, media prorenin concentrations in response to incubation with 100 μM dobutamine or terbutaline relative to controls as measured at 6-hr intervals. *Bottom*, tissue concentrations of prorenin after incubation with 100 μM dobutamine or terbutaline. Values represent mean ± standard error of six replicates from four individual placentas.

sponse to dobutamine and terbutaline (172 ± 15 and 73.8 ± 8.2 μunits/mg of protein, respectively) occurred after 24 hr of incubation. Tissue prorenin concentrations in control explants remained unchanged throughout the incubation period and were similar to those measured in nonincubated tissues (3.2 ± 0.3 μunits/mg of protein) despite an increase in the release of prorenin (Fig. 2, *bottom*). In general, the tissue prorenin content in the presence of the β-adrenergic agonists was increased, with the amount released being ~10–20% of the medium prorenin values.

Metabolic inhibitor effects on β-adrenoceptor activation-mediated prorenin synthesis and secretion. To examine the cellular mechanisms responsible for placental explant prorenin secretion in response to β-adrenoceptor activation, we used metabolic inhibitors (Table 1). Cycloheximide, which inhibits translational activity, was used to determine whether protein synthesis is necessary for the prorenin secretory response, whereas renin gene transcriptional activity was evaluated using actinomycin D, which intercalates into DNA indirectly inhibiting RNA polymerase. The effects of these inhibitors were measured after 24 hr of incubation. A concentration-dependent inhibition of the β-adrenergic agonist-induced increased prorenin secretion and tissue content was observed with both cycloheximide and actinomycin D.

Semiquantitative assessment of renin mRNA. The relative degree of renin gene expression after β-adrenoceptor activation was assessed by determining renin mRNA using semiquantitative and quantitative methods. Renin mRNA was semiquantitatively assessed using RT-PCR protocols designed for coamplification of renin cDNA with that of GAPDH. The relative amounts of renin and GAPDH cDNA were determined from Southern blot analysis.

The concentration-response effect of β-adrenoceptor activation of placental renin mRNA expression was evaluated (Fig. 3, *top*). The staining intensity of the bands corresponding to renin cDNA was greater in response to increasing concentrations of dobutamine. Southern blot analysis of renin and GAPDH cDNA from human placental explants incubated with dobutamine and terbutaline was evaluated for concentration-response relationships. There was little change in signal intensity for the target cDNA with concentrations of dobutamine of <10 μM. Renin mRNA relative to

TABLE 1

Effect of cycloheximide and actinomycin D on placental prorenin

Explants were incubated with cycloheximide or actinomycin D for 2 hr before the addition of terbutaline or dobutamine. Prorenin concentrations determined after explants were incubated for 24 hr. Results are the mean ± standard error of five experiments, each of which was replicated six times.

	Prorenin concentration (μunits/mg of protein)				
		Cycloheximide		Actinomycin D	
		1 μM	10 μM	1 μM	10 μM
Control					
Medium	10.8 ± 1.1	8.7 ± 0.5	1.2 ± 0.3[a]	5.4 ± 0.5[a]	1.0 ± 0.2[a]
Tissue	3.8 ± 0.5	2.9 ± 0.4	0.4 ± 0.1[a]	2.0 ± 0.4[a]	0.4 ± 0.1[a]
Terbutaline 100 μM					
Medium	63.5 ± 5.7	41.2 ± 5.1[a]	13.4 ± 1.3[a]	22.1 ± 2.5[a]	3.5 ± 0.5[a]
Tissue	9.2 ± 1.1	5.3 ± 0.5[a]	2.4 ± 0.4[a]	6.8 ± 2.0[a]	0.4 ± 0.1[a]
Dobutamine 100 μM					
Medium	182 ± 21	135 ± 18[a]	22.5 ± 4.8[a]	25.8 ± 4.1[a]	6.8 ± 1.3[a]
Tissue	15.6 ± 2.1	12.9 ± 1.8[a]	4.3 ± 0.8[a]	4.6 ± 0.9[a]	1.1 ± 0.3[a]

[a] Different from value in the absence of inhibitor as determined by analysis of variance, $p < 0.05$.

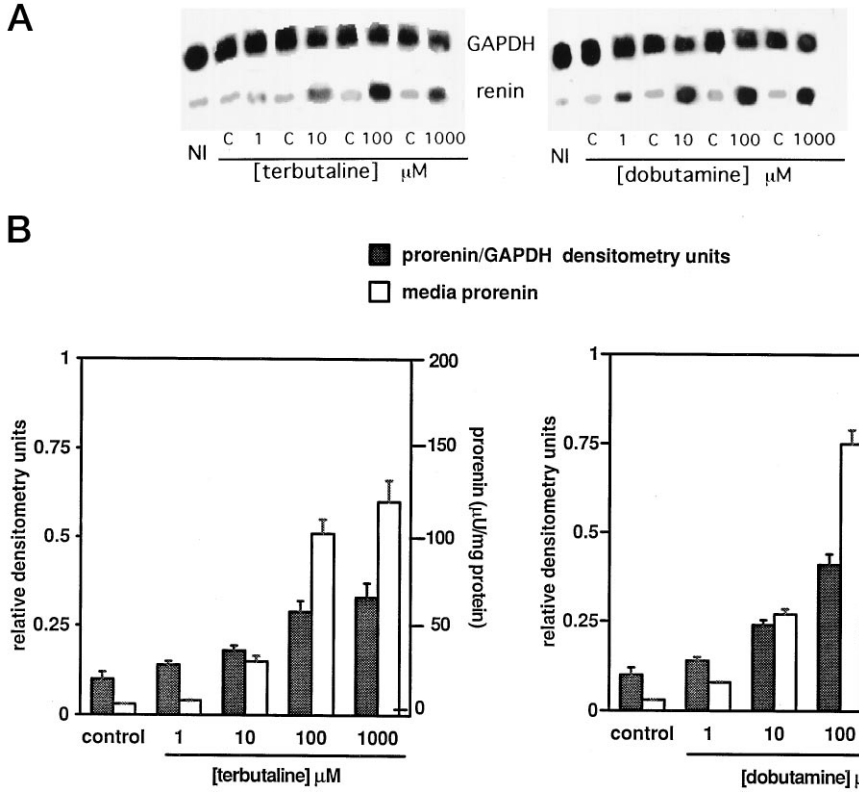

Fig. 3. Concentration-response effects of dobutamine and terbutaline on renin gene mRNA levels and prorenin medium concentrations. *Top*, representative Southern blot of renin and GAPDH cDNA for untreated control explants (*C*) and those treated with dobutamine or terbutaline (1–1000 μM) after 24 hr of incubation. Each analysis represents the pooling of six individual explants from the same tissue. *Bottom*, densitometry measurements of renin cDNA relative to GAPDH cDNA (relative densitometry units) are depicted with corresponding medium prorenin values.

GAPDH increased with higher concentrations of β-adrenoceptor agonist (Fig. 3, *bottom*).

Establishment of quantitative PCR for renin mRNA. The synthesis of internal control RNA (mutREN) was designed according to method of Wang (23) with modifications allowing the incorporation of GAPDH cDNA to distinguish the PCR products from those of the target mRNA. To determine the proper conditions for quantitative PCR, a number of parameters were tested. We examined various amplification cycles conducted with 2×10^7 molecules of control RNA and 1 μg of total RNA from placenta. The radioactivity incorporated into the PCR products was determined at sequential cycles (Fig. 4). It was demonstrated that the PCR remained in exponential phase through 26 cycles followed by a plateau in incremental amplification of the PCR products. Similarly, we also measured the incorporation of primers into amplicons generated from PCRs using various concentrations of unlabeled primers. Optimal incorporation was observed when 50 pmol of each primer was used. Various PCR buffers were used, and the optimal amplification occurred using 2.5 mM final $MgCl_2$ concentration. PCRs were conducted using various annealing temperatures and times, with 56° and 1 min being determined as optimal. The PCR assay was repeated using the same aliquots of RNA, which yielded an interassay variability of 4.5% (nine experiments).

Amplification was carried out with known concentrations of mutREN control RNA and total RNA, and the signal intensities of the representative PCR products were determined (Fig. 5A). To verify that the PCR amplicons represented mutREN and native renin cDNA, PCR was conducted with unlabeled primers and Southern hybridization was performed with a renin probe. The results demonstrated signals that paralleled the intensity of the amplicons corresponding

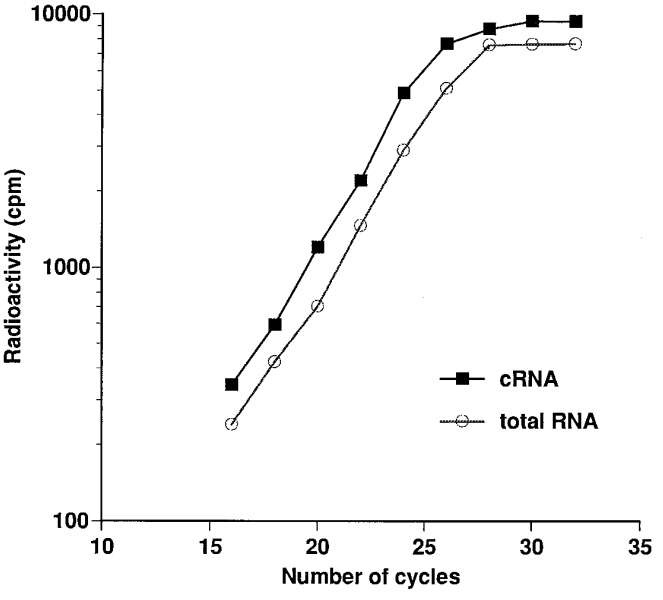

Fig. 4. Linearity of the PCR-amplification products of renin cDNA and mutREN relative to number of PCR cycles. Placental total RNA (1 μg) and 2×10^7 molecules of control RNA were reverse transcribed and used for sequential PCR amplification. Radioactivity of PCR products was assessed with a Molecular Dynamics PhosphoImager.

to the control RNA and target mRNA (Fig. 5B). Serial dilutions of template were used to generate linear regression equations from each curve (Fig. 5C). By using this procedure, numerical values of specific renin control RNA per unit of total RNA could be obtained for each tissue sample.

Quantification of placental explant renin mRNA. Placental explant renin mRNA were quantified before and

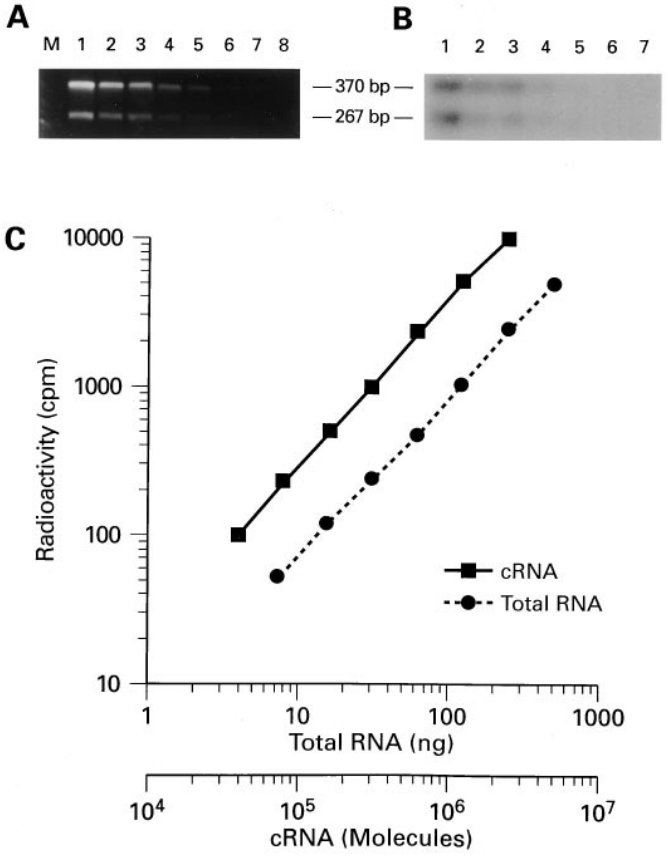

Fig. 5. Quantitative analysis of renin mRNA in placental explants. A, Ethidium bromide staining of PCR products separated on a 4% NuSieve/agarose (3:1) gel. *Lanes 1–7*, renin PCR products from serial 1:2 dilutions of a mixture containing 900 ng of total RNA and 2×10^6 molecules of mutREN control RNA after amplification for 25 cycles. *Lane 8*, control reaction without template and amplified for 25 cycles. The 267 bp of renin cDNA and 380 bp of mutREN are indicated. B, Characterization of the RT-PCR products by Southern analysis. Hybridization with renin probe, which is common to amplicons corresponding to renin cDNA and mutREN cDNA. C, Radioactivity from the bands in A were assessed with a Molecular Dynamics PhosphoImager. The variable template concentrations of the internal standard mutREN and placental total RNA were plotted against the radioactivity of their respective PCR products.

after incubation with dobutamine and terbutaline (Table 2). Renin mRNA values were increased after 6 hr of incubation, remained elevated after 24 hr, and were greater with dobutamine incubation. Measurements after 48 hr of incubation were in general near or below control levels. Tissues that were treated with 10 μM actinomycin D did not demonstrate increased renin mRNA in response to β-adrenoceptor agonists. Similar experiments conducted with 10 μM cycloheximide had no effect on the enhanced renin mRNA observed with dobutamine or terbutaline treatment.

Discussion

Several extrarenal RASs have been described in which the regulation of prorenin synthesis differs from that found in the kidney (24). Evidence suggests that receptor-coupled cAMP accumulation in placental tissue is involved in prorenin secretion (7). A regulatory role of β-adrenoceptors in prorenin synthesis and secretion is supported by the abundance of β_1- and β_2-adrenoceptors in villous placenta (25, 26)

TABLE 2

Effects of β-adrenoceptor activation on renin mRNA in placental explants

Results are mean ± standard error. Three to six different experiments were conducted for each treatment from a minimum of three placentas to express renin mRNA levels by quantitative PCR.

Renin mRNA ($\times 10^5$) molecules/100 ng of total RNA			
Incubation time	6 hr	24 hr	48 hr
Control	0.5 ± 0.1	0.4 ± 0.1	0.2 ± 0.5
Terbutaline 10 μM	0.8 ± 0.2[a]	0.7 ± 0.2[a]	0.4 ± 0.6
Terbutaline 100 μM	1.7 ± 0.2[a]	1.6 ± 0.2[a]	0.6 ± 0.4
+ Cycloheximide 10 μM	1.7 ± 0.3	2.1 ± 0.2[a]	0.8 ± 0.3
+ Actinomycin D 10 μM	0.3 ± 0.1[b]	0.3 ± 0.1[b]	0.3 ± 0.3
Dobutamine 10 μM	2.6 ± 0.7[a]	2.4 ± 0.2[a]	0.2 ± 0.3
Dobutamine 100 μM	4.3 ± 0.8[a]	4.2 ± 0.2[a]	1.8 ± 0.8
+ Cycloheximide 10 μM	4.6 ± 0.8[a]	4.4 ± 0.8[a]	1.6 ± 0.5
+ Actinomycin D 10 μM	0.7 ± 0.1[a]	0.4 ± 0.1[a]	0.2 ± 0.5

[a] Significantly different from control values.
[b] Significantly different from corresponding treatment without cycloheximide or actinomycin D.

and by the discovery that activation of these sites modifies placental hormone secretion (27).

Previous studies have shown that placental prorenin secretion is enhanced by β-adrenoceptor activation through cAMP and cAMP-dependent kinase-mediated phosphorylation events (28). Among the possible mechanisms responsible for enhanced renin secretion in placental tissue is the release of stored prorenin in response to β-adrenoceptor activation, such as occurs in the kidney. Because the constitutive release of prorenin from villous placenta is very low, the release of a stored form of prorenin by a regulated pathway is plausible. However, storage granules containing prorenin or renin have not been identified in placenta. Furthermore, the relatively low concentrations of prorenin in villous placental tissue do not support this concept.

Several lines of evidence from the current study support the notion that enhanced prorenin secretion induced by β-adrenoceptor activation in the placenta is primarily due to an increased synthesis rather than mobilization from storage granules, as seen in the kidney. First, the earliest effect of β-adrenoceptor agonist on prorenin secretion was not observed until after 6 hr of incubation. Second, increased prorenin concentration in both medium and tissue was observed after stimulation with that in the corresponding explants representing only 10% of that in the medium, thus indicating that the prorenin secreted and that found in the tissues are dependent on newly synthesized protein. Third, prorenin secretion and tissue content resulting from β-adrenoceptor activation were markedly attenuated by cycloheximide, an inhibitor of prorenin synthesis, providing further evidence that newly synthesized prorenin is responsible for enhanced secretion. In addition, the prorenin released into the incubation medium during β-adrenoceptor activation far exceeds the amount found in control and freshly prepared tissues.

In the current study, RT-PCR was used to evaluate renin mRNA levels in response to β-adrenoceptor activation. The amplification was validated by monitoring the PCR products of mRNA for the GAPDH gene or the mRNA transcribed *in vitro* from a mutant renin cDNA. Both of these techniques have been used successfully to study β-adrenoceptor activation-associated gene transcription events and to evaluate renin mRNA levels in a number of tissues (29, 30). Activation

of villous placental β-adrenoceptors increased renin mRNA levels after 6 hr and preceded increases in prorenin tissue content and secretion. It was demonstrated that activation of β_1-adrenoceptors produced a greater effect than that of β_2-adrenoceptors, which is consistent with the pattern of prorenin synthesis and secretion observed here and in earlier studies (7). The selectivity of these responses is likely the result of the relative distribution of β-adrenoceptor subtypes in placental tissue (65% and 35% for β_1 and β_2, respectively) (26). The association of renin mRNA levels and enhanced prorenin secretion contrasts with observations in kidney tissue, in which elevated renin mRNA levels after β-adrenoceptor activation are not observed until well after the secretion of prorenin has occurred (31). It has been hypothesized that an increased level of transcription activity is necessary to replenish the depleted renal stores of the peptide (31). Although little stored prorenin is found in the villous placenta and no immediate secretory response occurs in response to β-adrenoceptor activation, the time necessary for increased renin mRNA in response to the β-adrenoceptor stimulation is similar to that found in kidney.

The enhancement of renin mRNA levels by β-adrenoceptor activation was unaffected by cycloheximide, suggesting that these transcription events are independent of protein synthesis and thus cannot account for the delay in prorenin secretion. In addition, the increased prorenin secretion occurred at a much greater magnitude than the enhancement of renin mRNA, indicating that the increased prorenin mRNA level is not the only factor responsible for enhanced secretion during β-adrenoceptor activation. This feature is not surprising given the complexity of renin gene regulation and protein processing, as has been found with respect to other proteins and their regulation (32). Furthermore, classic gene transcription-mediated events by the CRE/CREM system are more rapid than the 6–12-hr delay observed in kidney and placenta (33). The time course of enhanced prorenin synthesis is closely related to the increase in the level of mRNA encoding this polypeptide during β-adrenoceptor activation. It has been reported that cAMP-stimulated gene expression in chorionic cells is primarily due to increased renin gene transcription (8). In contrast, a study of juxtaglomerular granular cells demonstrated that cAMP selectively increased the stability of renin mRNA (12). Further evidence has suggested that the regulatory mechanisms for renin mRNA by cAMP in a pulmonary carcinoma cell line are independent of the classic CRE/CREB pathway and that post-transcriptional regulation may be mediated through a protein factor (13). Also, cAMP has been shown to enhance the binding of nuclear factors to promoter elements in human embryonic kidney 293 cells, thus regulating renin gene transcription (34). These apparent discrepancies may be related to the differences in cell types and transfection systems that were used in the studies. In this investigation, we have shown that renin mRNA levels are similar at 6 hr to those at 24 hr of β-adrenoceptor activation. This finding suggests that the effect of cAMP on renin gene expression is phase dependent in placental explants in that the transcription rate is higher than degradation during the first 6 hr of incubation and thereafter the rates are similar. Conclusive evidence supporting the alternative mechanisms of cAMP effects on renin mRNA cannot be drawn from these studies and requires further attention.

The pharmacological responses to β-adrenoceptor activation and cAMP-mediated prorenin synthesis and secretion in villous placenta differ from responses observed in other tissues. The results indicate that *in vitro* activation of villous placental β-adrenoceptors by selective β_1- and β_2-adrenoceptor agonists increases prorenin secretion in a time- and concentration-dependent manner. This contrasts with the kidney, in which such responses are observed only with β_1-adrenoceptor agonists (35). In addition, chorion cell culture preparations resulted in a 2-fold increase in prorenin release via activation of adenylate cyclase by forskolin (8). This differs in the magnitude of the response observed in this study and may be the result of variations in cAMP generation under these experimental conditions, influences of various cell types that exist in the explant preparation, and the consideration that additional cell types other than trophoblast may secrete prorenin on adrenoceptor activation.

The effects of β-adrenoceptor activation on prorenin synthesis may have additional importance in other tissues with local RASs. The unique limitation of higher angiotensin concentrations to the these tissues may enable regulation of cellular functions, including the inhibition of renin synthesis similar to renal negative-feedback mechanisms (36, 37). It should also be considered that the cAMP-mediated effects on the placental RAS regulation may not be limited to the effects on renin gene expression because the angiotensin II AT_1 receptor mRNA levels are decreased in response to adenylate cyclase stimulation in some tissues (38). The relationship between the renin and AT_1 gene transcription rates and overall activity of the placental RAS as a result of β-adrenoceptor activation has not been studied.

The responses examined here likely represent a component of a larger scale of regulatory phenomena that influence the placental RAS. Because prorenin may catalyze angiotensin I formation under some conditions (39), an understanding of its regulation may provide insight into the functions of a local RAS in reproductive biology. Regulation of prorenin synthesis by catecholamine activation of β-adrenoceptors may serve as a mechanism to influence these processes.

Acknowledgments

The scientific contributions to the project by Dr. Andrew Parkinson are gratefully acknowledged.

References

1. Wilkes, B. M., E. Krim, and P. F. Mento. Evidence for a functional renin angiotensin system in full-term placental unit. *Am. J. Physiol.* **249:**E366–E373 (1985).
2. Alhenc-Gelas, F., A. Tache, J. P. Saint-Andre, J. Milliez, C. Sureau, P. Corvol, and J. Menard. The renin-angiotensin system in pregnancy and parturition. *Adv. Nephrol.* **15:**25–33 (1986).
3. Glance, D. G., M. G. Elder, D. L. Bloxam, and L. Myatt. The effects of the components of the renin-angiotensin system on the isolated perfused human placental cotyledon. *Am. J. Obstet. Gynecol.* **149:**450–454 (1984).
4. Wilkes, B. M., and P. F. Mento. Bradykinin-induced vasoconstriction and thromboxane release in perfused human placenta. *Am. J. Physiol.* **254:** E681–E686 (1988).
5. Petit, A., G. Guillon, M. Tence, S. Jard, N. Gallo-Payet, D. Bellabarba, J. G. Lehoux, and S. Belisle. Angiotensin II stimulates both inositol phosphate production and human placental lactogen release from human trophoblastic cells. *J. Clin. Endocrinol. Metab.* **69:**280–286 (1989).
6. Le Noble, F. A. C., J. W. M. Hekking, H. W. M. Van Straaten, D. W. Slaaf, and H. A. J. Struyker Bouldier. Angiotensin II stimulates angiogenesis in the chorio-allantoic membrane of the chick embryo. *Eur. J. Pharmacol.* **195:**305–306 (1991).
7. Downing, G. J., A. M. Poisner, and R. Poisner. β-Adrenoceptor activation stimulates, and phosphodiesterase inhibitors potentiate, placental prorenin synthesis and release. *J. Clin. Endocrinol. Metab.* **78:**41–47 (1994).

8. Duncan, K. G., M. A. Haidar, J. D. Baxter, and T. L. Reudelhuber. Regulation of human renin expression in chorion cell primary cultures. *Proc. Natl. Acad. Sci. USA* **87:**7588–7592 (1990).
9. Sun, J., C. Oddoux, M. T. Gilbert, Y. Yan, A. Lazarus, W. G. Campbell, and D. F. Catanzaro. Pituitary-specific transcription factor (Pit-1) binding site in the human renin gene 5′-flanking DNA stimulates promoter activity in placental cell primary cultures and pituitary lactosomatotropic cell lines. *Circ. Res.* **75:**624–629 (1994).
10. Borensztein, P., S. Germain, S. Fuchs, J. Philippe, P. Corvol, and F. Pinet. *cis*-regulatory elements and *trans*-acting factors directing basal and cAMP-stimulated human renin gene expression in chorionic cells. *Circ. Res.* **74:**764–773 (1994).
11. Germain, S., T. Konoshita, J. Philippe, P. Corvol, and F. Pinet. Transcriptional induction of the human renin gene by cyclic AMP requires cyclic AMP response element-binding protein (CREB) and a factor binding a pituitary-specific trans-acting factor (Pit-1) motif. *Biochem. J.* **316:**107–113 (1996).
12. Chen, M., J. Schnermann, A. M. Smart, F. C. Brosius, P. D. Killen, and J. P. Briggs. Cyclic AMP selectively increases renin mRNA stability in cultured juxtaglomerular granular cells. *J. Biol. Chem.* **268:**24138–24144 (1988).
13. Lang, J. A., L. H. Ying, B. J. Morris, and C. D. Sigmund. Transcriptional and posttranscriptional mechanisms regulate human renin gene expression in Calu-6 cells. *Am. J. Physiol.* **271:**F94–F100 (1996).
14. Huot, R. I., J. M. Foidart, and K. Stromberg. Effects of culture conditions on the synthesis of human chorionic gonadotropin by placental organ culture. *In Vitro Cell. Dev. Biol.* **15:**497–502 (1979).
15. Poisner, A. M., and R. Poisner. The use of human chorionic membranes and isolated trophoblast for studying renin secretion, in *In Vitro Methods for Studying Secretion* (A. M. Poisner and J. M. Trifaro, eds.). Amsterdam, Elsevier, 155–169 (1987).
16. Kataoka, K., N. Kurokawa, C. Yanaihara, S. Takahara, A. Okuyama, and N. Yanaihara. Prorenin and renin in human tissues and plasma: immunochemical identification. *Immunopharmacology* **32:**146–148 (1996).
17. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:**248–254 (1976).
18. Chomczinski, P., and N. Sacchi. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162:**156–159 (1987).
19. Hobart, P. M., M. Fogliano, B. A. O'Connor, I. M. Schaefer, and J. M. Chirgwin. Human renin gene: structure and sequence analysis. *Proc. Natl. Acad. Sci. USA* **81:**5026–5030 (1984).
20. Ungerer, M., M. Bohm, J. S. Elce, E. Erdmann, and M. J. Lohse. Altered expression of β-adrenergic receptor kinase and β_1-adrenergic receptors in the failing human heart. *Circulation* **87:**454–463 (1993).
21. Jablonsky, E. Preparation of oligodeoxynucleotide-alkaline phosphatase conjugates and their use as hybridization probes. *Nucleic Acids Res.* **14:** 6115–6128 (1986).
22. Pollard-Knight, D., A. C. Simmonds, A. P. Schapp, H. Akhavan, and M. A. Brady. Nonradioactive DNA detection on Southern blots by enzymatically triggered chemiluminescence. *Anal. Biochem.* **185:**353–358 (1990).
23. Wang, A. M., M. V. Doyle, and D. F. Mark. Quantitation of mRNA by the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* **86:**9717–9721 (1989).
24. Griendling, K. K., T. J. Murphy, and R. W. Alexander. Molecular biology of the renin-angiotensin system. *Circulation* **87:**1816–1828 (1993).
25. Schocken, D. D., M. G. Caron, and R. J. Lefkowitz. The human placenta: a rich source of B-adrenergic receptors: characterization of the receptors in particular and soluble preparations. *J. Clin. Endocrinol. Metab.* **50:**1082–1088 (1980).
26. Bahouth, S. W., and C. C. Malbon. Human *beta*-adrenergic receptors: simultaneous purification of *beta* 1- and *beta* 2-adrenergic-receptor peptides. *Biochem. J.* **248:**557–566 (1987).
27. Caritis, S. N., A. P. Hirsch, and A. J. Zeleznik. Adrenergic stimulation of placental progesterone production. *J. Clin. Endocrinol. Metab.* **56:**969–972 (1983).
28. Downing, G. J., and A. M. Poisner. cAPK mediates placental renin secretion stimulated by β-adrenoceptor activation. *Am. J. Physiol.* **267:**E954–E960 (1994).
29. Gilliland, G., S. Perrin, and H. F. Bunn. Competitive PCR for quantitation of RNA, in *PCR Protocols: A Guide to Methods and Applications* (M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds). New York, Academic Press (1990).
30. Paul, M., J. Wagner, and V. J. Dzau. Gene expression of the renin-angiotensin system in human tissues. *J. Clin. Invest.* **91:**2058–2064 (1993).
31. Dzau, V. J., D. W. Burt, and R. E. Pratt. Molecular biology of the renin-angiotensin system. *Am. J. Physiol.* **255:**F563–F573 (1988).
32. Scarbrough, K., N. G. Weiland, G. H. Larson, M. A. Sortino, S. F. Chiu, A. N. Hirschfield, and P. M. Wise. Measurement of peptide secretion and gene expression in the same cell. *Mol. Endocrinol.* **5:**134–142 (1991).
33. Roesler, W. J., G. R. Vandenbark, and R. W. Hanson. Cyclic AMP and the induction of eukaryotic gene transcription. *J. Biol. Chem.* **263:**9063–9066 (1988).
34. Tamura, K., S. Umemura, S. Yamaguchi, T. Iwamoto, S. Kobayashi, A. Fukamizu, K. Murakami, and M. Ishii. Mechanism of cAMP regulation of renin gene transcription by proximal promoter. *J. Clin. Invest.* **94:**1959–1967 (1994).
35. Weber, F., O. E. Brodde, M. Anlauf, and K. D. Bock. Subclassification of human *beta*-adrenergic receptors mediating renin release. *Clin. Exp. Hypertens. Part A Theory Pract.* **A5:**225–238 (1983).
36. Poisner, A. M., G. J. Downing, and R. Poisner. Prorenin secretion from villous placenta: regulation by cyclic AMP and angiotensin. *Placenta* **15:** 487–499 (1994).
37. Johns, E. W., M. J. Peach, R. A. Gomez, T. Inagami, and R. M. Carey. Angiotensin II regulates renin gene expression. *Am. J. Physiol.* **259:**F822–F887 (1990).
38. Lassègue, B., R. W. Alexander, G. Nickenig, M. Clark, T. J. Murphy, and K. K. Griendling. Angiotensin II down-regulates the vascular smooth muscle AT_1 receptor by transcriptional and post-transcriptional mechanisms: evidence for homologous and heterologous regulation. *Mol. Pharmacol.* **48:**601–609 (1995).
39. Edalji, R., T. F. Holzman, and E. J. Gubbins. Active prorenin: evidence for the formation of a conformational variant of recombinant human prorenin. *J. Protein Chem.* **10:**403–406.

Send reprint requests to: Dr. Gregory Downing, Endocrinology and Reproduction Research Branch, NICHHD, Bldg. 49, Room 6A35, 9000 Rockville Pike, Bethesda, MD 20892-4510.