There is no definitive statement of the precision of the broadcast ephemerides. Our experience suggests that they are accurate at about the 5-m level, or ~0.25 ppm. We obtained the same results (at the millimeter level) when we processed selected Parkfield observations with improved orbits.

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Novel Sites of Expression of Functional Angiotensin II Receptors in the Late Gestation Fetus

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In the adult, the peptide hormone angiotensin II (AII) is primarily known as a regulator of circulatory homeostasis, but recent evidence also suggests a role in cell growth. This study of AII in late gestation rat fetuses revealed the unexpected presence of receptors in skeletal muscle and connective tissue, in addition to those in recognized adult target tissues. The AII receptors in this novel location decreased by 80 percent 1 day after birth and were almost undetectable in the adult. Studies in fetal skin fibroblasts showed that the receptors were coupled to phospholipid breakdown, with concomitant increases in inositol phosphate and cytosolic calcium. The abundance, timing of expression, and unique localization of functional AII receptors in the fetus suggest a role for AII in fetal development.

EMBRYOGENESIS, FETAL DEVELOPment, and growth are controlled by the coordinated action of a number of humoral regulators. In addition to traditional growth factors (*1*), an increasing number of peptide hormones have been implicated in cellular growth regulation (*2*). The octapeptide angiotensin II (AII), classically associated with control of blood pressure and electrolyte metabolism (*3*), stimulates cell growth and increases the expression of platelet-derived growth factor (PDGF) and the growth-related proto-oncogenes c-*myc* and c-*fos* in cultured smooth muscle cells (*4, 5*). Abundant binding sites for AII are present in membranes prepared from eviscerated rodent fetuses, indicating the presence of AII receptors at sites other than the traditional target in the adult (*6*). All components of the renin-angiotensin system, including immunoactive and bioactive AII, are found in the fetal-placental unit, thus providing the specific ligand for the fetal AII binding sites (*7*).

To characterize the topographic distribution of AII receptors in the fetus, we obtained female Sprague-Dawley rats at 7 to 21 days of gestation, litters of different ages, and adults from Zivic Miller (Zelienople, PA). Rats were killed and fetuses were immediately removed and frozen at −40°C. Autoradiographic analysis of AII receptors in the fetuses was performed by binding of ^{125}I-labeled [Sar^1,Ile^8]AII to slide-mounted sections (*8*).

Midsagittal and lateral sagittal autoradiograms from a 19-day-old fetus are shown in Fig. 1, A and B, respectively. All organs are fully formed at this stage, and AII binding was readily detectable in known target tissues including the adrenal zona glomerulosa and medulla, kidney, liver, and smooth muscle of the bronchi, blood vessels, and gastrointestinal tract. Particularly striking, however, is the intense AII binding in areas not normally expected to contain AII receptors, such as the subepidermal layer of the skin, mesenchymal and connective tissues, and skeletal muscle, especially the tongue. Little or no binding was detectable by autoradiography in brain, spinal cord, cartilage, bone, fat, and heart. The observed binding was specific, since all staining was abolished by incubation in the presence of 1 μ*M* unlabeled AII, but was unchanged by excess amounts of the unrelated peptides, corticotropin-releasing factor (CRF), arginine vasopressin (AVP), and adrenocorticotropic hormone (ACTH).

AII binding was first detected at about day 12, and by the time organogenesis was completed (day 15), its localization and density was as described in Fig. 1. The high receptor density seen by autoradiography

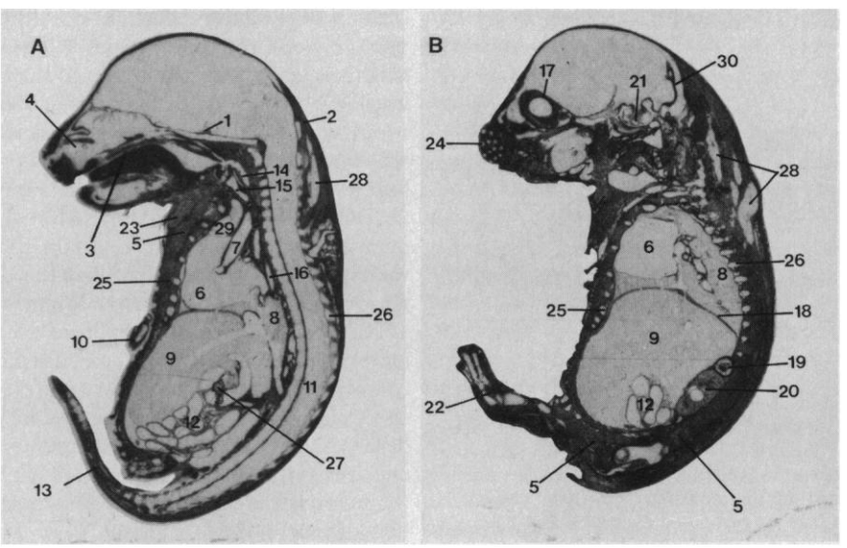

Fig. 1. Autoradiographic analysis of ^{125}I-labeled [Sar^1,Ile^8]AII binding to 20 μm of sagittal frozen sections of 19-day-old rat fetus (**A**) medial section (**B**) lateral section. Nonspecific binding measured in the presence of 1 μ*M* AII was undetectable (not shown). Topographic distribution of the binding as determined by light microscopic analysis of the sections is indicated by the numbers: 1, pituitary gland; 2, mesenchymal tissue; 3, tongue; 4, nasal cavity; 5, skeletal muscle; 6, heart; 7, aorta; 8, lung; 9, liver; 10, umbilical cord; 11, spinal cord; 12, small bowel; 13, tail; 14, esophagus; 14, larynx; 16, trachea; 17, soft tissue around the eye; 18, diaphragm; 19, adrenal gland; 20, kidney; 21, inner ear; 22, hind foot; 23, salivary gland; 24, analgene of vibrissae; 25, rib; 26, vertebral bodies; 27, root of mesentery; 28, brown fat deposit; 29, thymus; and 30, meninges. The figure is representative of five similar experiments.

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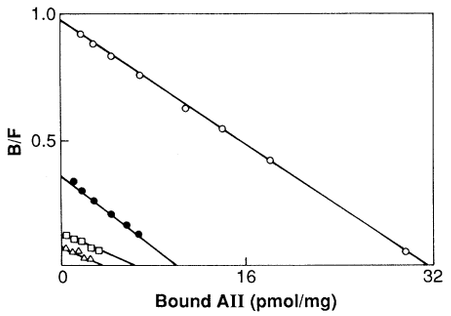

Fig. 2. Changes in AII receptor concentration in skin during development as shown by Scatchard analysis of the binding data in membrane-rich fractions. Points are the means of duplicate incubations in a representative experiment. B/F, Bound to free ratio. Fetus (○), 1 (●), 7 (□), and 14 (△) days after birth. The data are representative of three similar experiments.

was maintained from day 18 to 21 of gestation, but within 1 day of birth showed a dramatic overall decrease. Quantitation of the decrease in AII receptors after birth was performed by binding of ^{125}I-labeled [Sar^1, Ile^8]AII to membranes from skin and lingual skeletal muscle, tissues that contain the highest concentration of binding sites in the fetus. AII receptors in skin membranes were reduced only 1 day after birth by 80% compared with the 19-day fetus (Scatchard plots, Fig. 2) (44,400 ± 8,980, n = 5, and 9,110 ± 480 fmol/mg, n = 3, mean ± SD). Receptor content continued to decrease to 8.8% and 6% at 7 and 14 days after birth and became undetectable in the adult. The affinity (K_d) of the AII receptor was $6.2 \times 10^{-9}M$ in fetal skin and remained at similar values after birth. In the tongue, used as a representative tissue for skeletal muscle, AII receptor content also decreased after birth but to a lesser extent compared with skin (24,430 ± 7,390 fmol/mg in the fetus to 11,390 ± 1,390, and 3,930 ± 190 fmol/mg at 5 and 14 days after birth, respectively, and was still detectable in the adult, 46 ± 2.4 fmol/mg, mean ± SD, n = 3). The dramatic decrease in AII receptor concentration in skin and skeletal muscle after birth was in contrast to the persistent high receptor levels observed in vesicular smooth muscle, adrenal, kidney, and pituitary, known target tissues for AII in the adult (*9*).

To determine whether these unique fetal AII binding sites are functional receptors, we investigated their coupling to intracellular messenger systems using cultured cells prepared from fetal skin. Binding and activation experiments were performed on first and second passage cultures, which on light microscopic examination appeared as a homogeneous cell population with fibroblast-like characteristics (*10*). These cells contained abundant AII receptors with affinity and specificity identical to those in eviscerated fetuses (*6*), fetal skin and skeletal tissue membranes and primary fetal skin cultures, thus providing a model to study functional aspects of the fetal AII receptor. Scatchard analysis of the binding data in three experiments revealed a single class of receptors with a K_d of $2.2 \pm 1.1 \times 10^{-9}M$ and a binding capacity of 483 ± 15 fmol per 10^6 cells (mean ± SD). The properties of these receptors were similar to those described in recognized AII target tissues (*3*).

The cultured skin fibroblasts were used to investigate whether occupancy of fetal AII receptors results in phospholipid breakdown and calcium mobilization (*11*), as occur in other target tissues (*12, 13*). Endogenous phosphoinositide pools were labeled by preincubation of the cells for 18 hours with [^{3}H]inositol and incubated with AII. As shown in other systems (*13*), treatment of the cells with 10 nM AII resulted in rapid increases in inositol 1,4-bisphosphate [Ins(1,4)P_2] and inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] as determined by high-performance liquid chromatography (HPLC) (Fig. 3). Ins(1,4,5)P_3 attained peak levels in 10 s and decreased to control values by 5 min, consistent with rapid metabolism to higher and lower inositol phosphates (*14*). Ins(1,4)P_2 also reached peak levels in 10 s, but declined more slowly, remaining elevated at 300% above the basal value at 10 min.

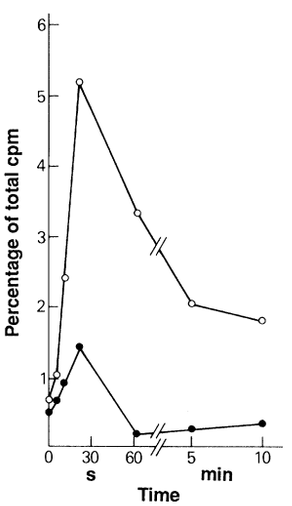

Fig. 3. Stimulation of inositol phosphate formation of AII in cultured fetal skin fibroblasts. Cells were prepared by trypsin digestion of dorsal skin of 18-day-old rat fetuses as previously described for rat pituitary cells (*9*). Cultures at the first or second passage were prelabeled for 18 hours with [^{3}H]inositol and stimulated with 10 nM AII for the times indicated, and inositol phosphate metabolites were analyzed by HPLC as previously described (*14*). Ins(1,4)P_2, inositol 1,4-bisphosphate (○); Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate (●). Similar results were obtained in two additional experiments.

Since Ins(1,4,5)P_3 formation results in calcium mobilization from intracellular stores associated with the endoplasmic reticulum (*11*), the effect of AII on cytosolic calcium was measured in cultured fetal fibroblasts with the fluorescent indicator, Fura-2 (*15, 16*). Perfusion of Fura-2–loaded cells with 10 nM AII resulted in a rapid increase in fluorescence 20 s after the levels of AII started to rise, and reached maximum after 100 s of exposure to the peptide (Fig. 4A). The calculated concentration of AII in the cuvette was 1 nM at the time of the initial increase in fluorescence and 5 nM at the peak. This effect of AII was completely abolished by simultaneous perfusion with the AII antagonist [Sar^1,Ile^8]AII (1 μM) commencing 5 min before exposure of the cells to AII (Fig. 4B).

The demonstration that AII increases inositol phosphate formation and cytosolic calcium indicates that the fetal AII binding sites are functional receptors for the peptide. Since the calcium-phospholipid pathway has been proposed as a common route in the

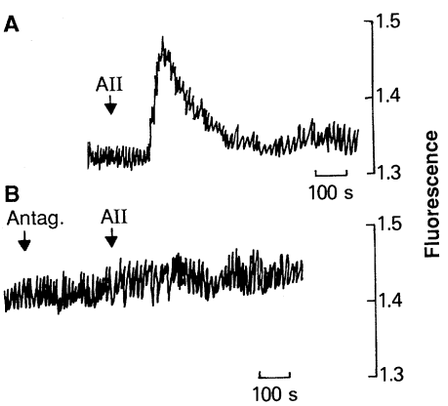

Fig. 4. Changes in cytosolic calcium in fetal skin fibroblasts perifused with AII in the absence (**A**) and in the presence (**B**) of 1 μM [Sar^1, Ala^8]AII commenced 250 s before addition of AII. Intracellular calcium was measured with the fluorescent indicator Fura-2. Second passage fetal cells were plated on glass cover slips (13 × 10 mm, no. 1, Corning) coated with 0.05% poly-D-lysine (Sigma). At 90% confluency cells were incubated in fresh media containing 1 μM Fura-2 AM for 30 min, washed twice in assay buffer (Medium 199 containing 25 mM Hepes and 0.01% bovine serum albumin) and kept on ice, in the dark, until intracellular calcium was assayed. Fluorescence was measured under dual excitation with wavelengths of 340 and 390 nm in a spectrofluorometer (8000C SLM Instruments, Urbana, Illinois) with a thermostatically controlled cuvette holder. The cover slip was inserted diagonally in a quartz cuvette modified from that described for muscle cells (*15*), in which two tubes were attached to enable perfusion of the cells at a rate of 1 ml/min with assay buffer containing reagents. The concentration of AII in the cuvette was calculated in separate experiments from the fluorescence measurement during perifusion with calcium-saturated Fura-2. The data are representative of three similar experiments.

action of several mitogens (*2, 17*), AII may be implicated in fetal growth. In support of this possibility, AII has been shown to act as a mitogen in cultured bovine adrenal cells (*18*) and to stimulate growth and induce the expression of c-*myc* and c-*fos* in cultured smooth muscle cells (*4, 5*). In addition, injection of AII in 18-day rat fetuses in utero caused a 20% increase in ^{3}H-labeled amino acid incorporation into proteins in the skin, but not in the brain, measured 5 hours later (*19*).

AII may modulate growth through its effect on the calcium-phospholipid pathway directly or through the regulation of the expression of other growth factors or proto-oncogenes. The latter is supported by the finding that AII causes an increase in PDGF mRNA in cultured smooth muscle cells (*5*). In addition, our findings on the ontogeny and localization of AII receptors are similar to those reported for insulin-like growth factor II in the rat fetus (*20*). Although the exact physiological function of AII in the fetus remains to be demonstrated, the prominent and transient expression of functional AII receptors at unique sites in the fetus strongly suggests a role for AII in fetal development.

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Recombinant Gene Expression *in Vivo* Within Endothelial Cells of the Arterial Wall

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A technique for the transfer of endothelial cells and expression of recombinant genes in vivo could allow the introduction of proteins of therapeutic value in the management of cardiovascular diseases. Porcine endothelial cells expressing recombinant β-galactosidase from a murine amphotropic retroviral vector were introduced with a catheter into denuded iliofemoral arteries of syngeneic animals. Arterial segments explanted 2 to 4 weeks later contained endothelial cells expressing β-galactosidase, an indication that they were successfully implanted on the vessel wall.

REDUCTIONS IN BLOOD FLOW TO the heart and other organs are often caused by fixed atherosclerotic obstructions, which can become critical when accompanied by superimposed narrowings due to thrombus formation or vessel constriction. Vascular endothelial cells contribute to the pathogenesis of these lesions by (i) altering the thrombogenic properties of the vessel lumen (*1*), (ii) inducing smooth muscle cell proliferation (*2*), and (iii) regulating vascular smooth muscle tone (*3*). Genetically altered endothelial cells could transmit recombinant DNA products that would provide anticoagulant, vasodilatory, angiogenic, or growth factors to a localized segment of vessel. We show that endothelial cells can be stably implanted in situ on the arterial wall and express a recombinant marker protein, β-galactosidase, in vivo.

Because atherogenesis in swine has similarities to humans, we used an inbred pig strain, the Yucatan minipig (Charles River Laboratories), as an animal model (*4*). We established a primary endothelial cell line from the internal jugular vein of an 8-month-old female minipig and confirmed the endothelial cell identity of this line (*5*). Two independent β-galactosidase–expressing endothelial cell lines were isolated (*6*) after they were infected with a murine amphotropic β-galactosidase–transducing retroviral vector (BAG), which is replication-defective and contains both β-galactosidase and neomycin resistance genes (*7*).

Endothelial cells derived from this inbred strain, being syngeneic, were applicable for study in more than one minipig, and were tested in nine different experimental subjects. The animals were anesthetized, the femoral and iliac arteries were exposed, and a catheter was introduced into the vessel through a small branch (Fig. 1). Intimal tissues of the arterial wall were denuded mechanically by forceful passage of a partially inflated balloon catheter within the vessel. The artery was rinsed with heparinized saline and incubated with a neutral protease dispase (50 U/ml), which removed any remaining luminal endothelial cells. Residual enzyme was presumably inactivated by α_2 globulin in plasma (*8*) on deflating the catheter balloons and allowing blood to flow through the vessel segment. The cultured endothelial cells, which expressed β-galactosidase (Fig. 2, A and B), were introduced by means of a specially designed arterial catheter (C. R. Bard Inc., Billerica, MA) that contained two balloons and a central instillation port (Fig. 1). When these balloons were inflated, a protected space was created within the artery into which cells were instilled through the central port. These β-galactosidase–expressing endothelial cells were allowed to incubate for 30 min to facilitate their attachment to the denuded vessel. The catheter was then removed, the arterial side branch ligated, and the incision closed. Labeling with ^{51}Cr indicated that, of the 2×10^5 endothelial cells instilled in the central space, 2 to 11% successfully attached to the denuded arterial wall.

Segments of experimental and control arteries were removed 2 to 4 weeks later. Staining of arterial specimens with X-gal chromagen revealed multiple areas of blue

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