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Ontogeny of Atrial Natriuretic Factor Receptors and Cyclic GMP Response in Rabbit Renal Glomeruli

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ABSTRACT. Atrial natriuretic factor (ANF) has been identified in fetal and newborn mammals, and considerable data regarding fetal ANF metabolism are available. However, there is limited information concerning ANF receptors or receptor ontogenesis in developing mammals. We measured ANF receptor binding capacity, affinity, and ANF-induced cyclic GMP (cGMP) generation in isolated renal glomeruli from fetal (29 d gestation, term = 31 d), newborn (3 d), juvenile (28 d), and adult rabbits. The (mean ± SEM) glomerular receptor binding capacity values for ANF in fetal and newborn animals (10 ± 1 and 12 ± 3 fmol/mg protein) were similar and significantly lower than the values for juvenile and adult animals (30 ± 8 and 74 ± 15 fmol/mg protein, respectively). In contrast, there were no significant differences in ANF receptor affinity values or dose-dependent increases in ANF-stimulated cGMP generation among the age groups studied. In competition studies, we observed effective displacement of ^{125}I-ANF by C-ANF$_{4-23}$, a ring-deleted ANF analogue, in adult, juvenile, and newborn glomeruli; however, C-ANF displaced only about 50% of the ^{125}I-ANF in fetal tissue. The addition of C-ANF did not elicit cGMP generation, nor did C-ANF affect ANF-induced cGMP generation in fetal, newborn, or adult glomeruli. These results indicate that *1*) the ANF receptor-guanylate cyclase system is intact in 29-d fetal rabbit glomeruli, and *2*) the ANF-induced cGMP formation is similar in fetal and adult animals, whereas receptor binding capacity is relatively higher in adult glomeruli. These results suggest a higher proportion of nonguanylate cyclase-coupled ANF receptors in the mature rabbit. (*Pediatr Res* 30: 45–49, 1991)

Abbreviations

ANF, atrial natriuretic factor
cGMP, cyclic GMP
B_{max}, receptor binding capacity
K_d, receptor affinity

ANF is a cardiac hormone with potent natriuretic, diuretic, and vasodilatory actions in adult animals and humans (1, 2). The actions of ANF are mediated through specific high-affinity receptors located in various tissues, including several renal structures, vascular endothelium, adrenal glomeruli, lungs, CNS, and the placenta (3–9). Autoradiographic studies of radiolabeled ^{125}I-ANF binding have demonstrated a high density of ANF binding sites in renal glomeruli (4). These glomerular ANF receptors have been shown to be functionally coupled to guanylate cyclase; ANF binding increases intracellular cGMP production, the second messenger that mediates most of the biologic effects of ANF (10). A second class of ANF receptors has been described in vascular and kidney tissues. These receptors are not linked to guanylate cyclase and have a suggested role in ANF clearance/storage from the circulation (11–13). Recently, activation of nonguanylate cyclase-coupled receptors has been found to increase inositol phosphates (14) and inhibit adenylate cyclase activity (15).

Immunoreactive ANF is present in mammalian fetal cardiac tissue early in gestation (16, 17), and relatively high ANF concentrations have been reported in the fetal circulation (18). However, the diuretic and natriuretic effects of ANF appear to be obtunded in immature animals relative to adults (19, 20). Recently, ANF receptors have been identified in fetal renal structures of the rat, and ANF binding capacity in these structures has been shown to increase with maturation (21–23). Thus, a reduced ANF binding capacity may account for the decreased action of ANF in immature kidneys, and immaturity of the fetal receptor-guanylate cyclase coupling system may contribute to the decreased action. Our study was designed to characterize the ontogenesis of renal glomerular ANF receptor binding and cGMP generation in the developing rabbit.

MATERIALS AND METHODS

Kidneys were obtained from fetuses at 29 d (term = 31 d) gestation (n = 28), newborn rabbits at 3 d (n = 21), juvenile animals at 4 wk of age (n = 6), and adult does (n = 6) immediately after delivery. Time-dated does (n = 6) were anesthesized with i.v. ketamine (50 mg/kg) and given oxygen (5 L/min) by face mask. After s.c. lidocaine (1%) administration, a midline abdominal incision was made. Fetal rabbits were removed from the uterus and killed immediately by decapitation. After removal of the last fetus, the doe was killed with an administration of pentobarbital (100 mg/kg). Three-d and 4-wk-old rabbits were anesthesized with an intramuscular injection of ketamine (50 mg/kg) and killed by decapitation.

Rabbit glomeruli were prepared by graded sieving as described by Carrier *et al.* (3). Each kidney was excised from the renal capsule and dissected longitudinally, and the cortical tissue was minced and homogenized with a tissue grinder. The minced tissue was gently passed through stainless steel sieves with decreasing mesh sizes of 200, 125, and 50 μm. The glomerular

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preparation was centrifuged (200 rpm for 3–5 min), and the pellet was resuspended in 50 mM Tris-HCl buffer, pH 7.4. Tubular fragments and blood cells represented <10% of the preparation, and unencapsulated glomeruli represented 80 to 85% of the total glomerular population. Protein concentration was determined using the Bradford method (24) with BSA as the standard. An aliquot of the fresh glomerular membrane preparation was used to assess cGMP production. The remainder was frozen at −70°C until it was used in receptor binding studies.

Radioreceptor assay. Synthetic human ANF (ANF_{99-126}) (Bachem Inc., Torrance, CA) was iodinated using sodium ^{125}iodide and iodogen (25). Iodinated peptide was purified on a Sep-pak C-18 column and eluted with a linear gradient of acetonitrile in 0.1% tetrafluoroethylene. Radioactivity in 10-μL portions of each fraction counted in a gamma counter, identified two peaks of activity. Measurement of sp act (approximately 1000–2000 Ci/mmol, respectively) determined by self-displacement (26) indicated that these peaks represented monoiodinated and diiodinated peptides.

The binding assay was performed in duplicate in 12 × 75 mm borosilicate glass tubes. Aliquots (50 μg of protein) of glomerular membranes were incubated for 30 min at 4°C in assay buffer (50 mM Tris-HCl, pH 7.4, 1 μM aprotinin, 0.1% bacitracin, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM $MgCl_2$, 0.4% BSA) containing ^{125}I-ANF (30 pM) and increasing concentrations (10^{-13} to 10^{-7} M) of unlabeled ANF in a final volume of 0.5 mL. Separation of the bound and free hormones was achieved by rapid filtration through 0.3% polyethylenimine-treated Whatman GF/C filters. The filters were washed three times with 3 mL of 50 mM Tris-HCl (pH 7.4), allowed to dry, and counted in an LKB gamma counter (LKB Instruments, Gaithersburg, MD). Competition studies also were conducted with increasing concentrations of AVP, angiotensin II, and a ring-deleted ANF analog, $C\text{-}ANF_{4-23}$ (Bachem Inc.). Saturation studies were conducted using increasing amounts of ^{125}I-ANF (10–400 pM). Specific binding was calculated as the difference between total binding and nonspecific binding determined in similar samples in the presence of excess unlabeled ANF (10^{-6} M).

Cyclic nucleotide determinations. Freshly prepared intact glomeruli (100 μg protein) were resuspended in Hanks' balanced salt solution at pH 7.4 and preincubated at 37°C. Two min after the addition of isobutylmethyxanthine at 0.5 mM final concentration, cGMP production was initiated by the addition of an increasing dose of ANF and/or C-ANF (at 10^{-9}–10^{-7} M, final concentration). The reaction was stopped after 5 min by adding 0.5 mL trichloroacetic acid (12 mg/dL). Precipitated protein was sedimented at 2000 × *g* for 15 min, the supernatant was extracted with ether, and cGMP concentrations were determined by RIA after acetylation (New England Nuclear Corp, Wilmington, DE).

Results are presented as the mean ± SEM for multiple experiments. Unpaired *t* test was used to determine statistically significant differences between the two groups at a significance level of $p < 0.05$. Competitive binding data were transformed by Scatchard analysis, using the LIGAND program (27).

RESULTS

Scatchard analysis of representative competitive binding data for ^{125}I-ANF binding to glomeruli from rabbit glomerular membranes indicated the presence of a single class of high-affinity binding sites from each age group (Fig. 1). Displacement of labeled ANF (30 pM) with unlabeled ANF was similar in fetal, newborn, juvenile, and adult glomerular membranes (Fig. 2). $C\text{-}ANF_{4-23}$ effectively displaced ^{125}I-ANF from binding sites in adult, juvenile, and newborn glomeruli. However, only 50% of the radiolabeled ligand was displaced in fetal tissue by C-ANF (Fig. 2). No displacement of labeled ANF was observed with unrelated compounds, such as AVP or angiotensin II, in any age group.

Table 1 lists the K_d and B_{max} values for ANF at the various developmental stages studied. In both fetal and newborn glomeruli, the B_{max} was lower ($p < 0.05$) than in juvenile and adult rabbits. Although the mean fetal K_d values tended to be lower, the K_d values did not differ statistically among the groups studied.

Glomerular membrane cGMP generation data in response to an increasing dose of ANF and/or C-ANF are shown in Figure 3. Basal glomerular membrane cGMP formation was similar in all rabbit age groups; no differences in ANF-induced cGMP generation were observed. cGMP was not stimulated by C-ANF, and ANF-induced cGMP formation in fetal, newborn, or adult glomeruli was not inhibited by the presence of C-ANF (10^{-6} M).

DISCUSSION

Our results indicate that specific ANF receptors are present on fetal rabbit glomerular membranes at 29 d gestation (term = 31 d). In addition, the B_{max} for ANF binding in glomerular membranes increases 7-fold from the fetal to the adult age group. These results are in agreement with earlier data in rats (22, 23). Although ANF binding capacity was age-dependent, there were no differences in cGMP generation among the age groups studied. This observation contradicts the previously reported correlation between ANF binding capacity and cGMP production in vascular (28) and glomerular membranes (29). However, Ballerman *et al.* (4) and Michel *et al.* (30) found no change in ANF-induced cGMP production from down-regulated ANF binding sites in salt-loaded rats. Thus, the significance of the observed dissociation of ANF receptor number and guanylate cyclase responsiveness is unclear but may reflect differences in receptor types.

The ANF receptor is a cell surface membrane-bound guanylate cyclase enzyme. Binding of ANF to an extracellular site on the enzyme elicits intracellular production of cGMP as a second messenger (10). The receptor exists in several forms, including a 120- to 135-kD single protein and a 66- to 70-kD truncated form lacking most of the intracellular domain (11, 31). The former binds the major circulating form of ANF (ANF_{99-126}), and binding evokes cGMP production. The truncated receptor accepts a wider range of ANF structures, but ligand binding does not evoke cGMP generation (30, 32). The truncated ANF receptor appears to be the predominant receptor in kidneys and is postulated to modulate ANF clearance (12, 32, 33). However, activation of nonguanylate cyclase-coupled receptors has been reported to induce formation of inositol phosphates (14), possibly regulating calcium mobilization, protein kinase C activity, and inhibition of the adenylate cyclase/cAMP signal transduction system (15). Additional studies are needed to establish which effects will be mediated through the occupancy of each of the ANF receptors.

In the present study, the truncated ANF analogue ($C\text{-}ANF_{4-23}$) bound to renal glomerular membranes in all age groups studied and, as expected, failed to elicit cGMP generation. That C-ANF binds to over 90% of adult renal ANF binding sites (12) is consistent with our observations and suggests that the majority of glomerular ANF binding sites represent non-cGMP coupled receptors in adults. C-ANF appeared to displace ^{125}I-ANF less readily from fetal glomeruli, although receptor density was substantially less than that in adult tissue. The response of cGMP to ANF in the rabbit glomeruli was similar to results obtained by other investigators (4, 15, 28–30). Although our data demonstrate the presence of guanylate cyclase-linked receptors in fetal rabbit glomeruli, the maturational increase in B_{max} in the absence of changes in cGMP generation suggests that the ratio of truncated to cGMP-generating receptors increases with gestation. In support of this hypothesis, Almeida *et al.* (33) observed a decreased ANF plasma clearance rate in rats in the presence of C-ANF and postulated that truncated receptors are active in ANF binding and clearance. In contrast, we were unable to show an effect of C-ANF on ANF plasma clearance in the early 3rd trimester ovine fetus (34).

Robillard *et al.* (20) observed that the 128- to 139-d gestation

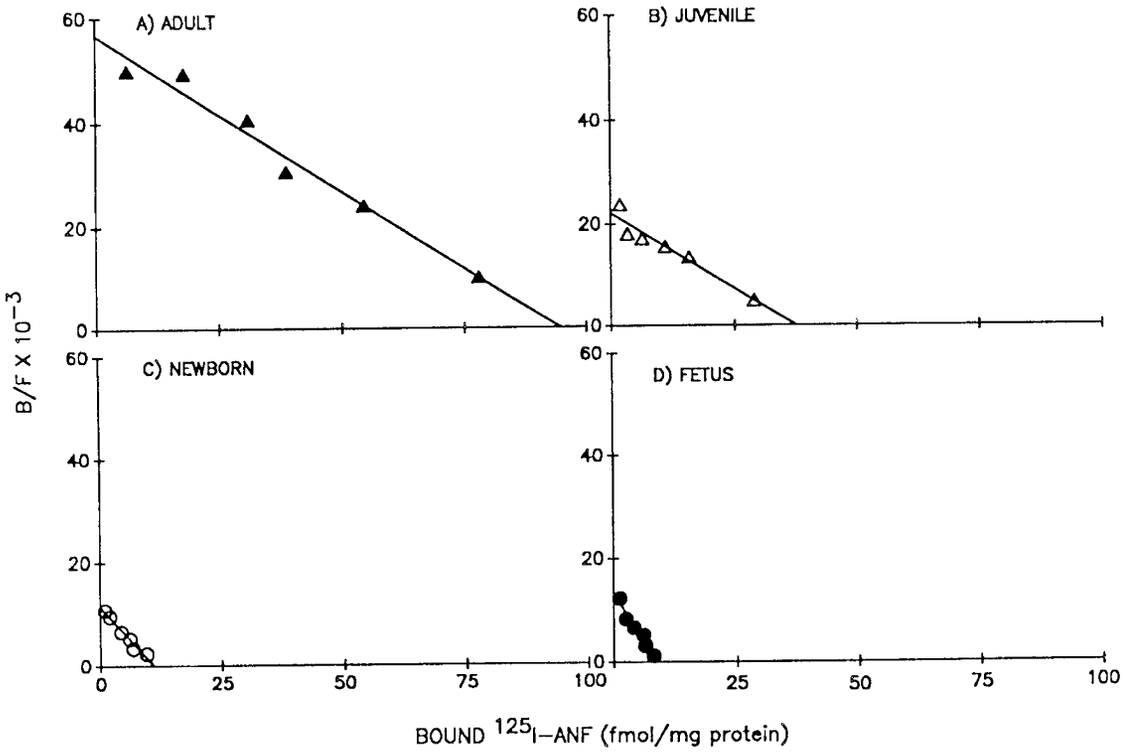

Fig. 1. Scatchard transformation of ^{125}I-ANF binding to *A*, adult; *B*, juvenile; *C*, newborn; and *D*, fetal rabbit glomerular membranes.

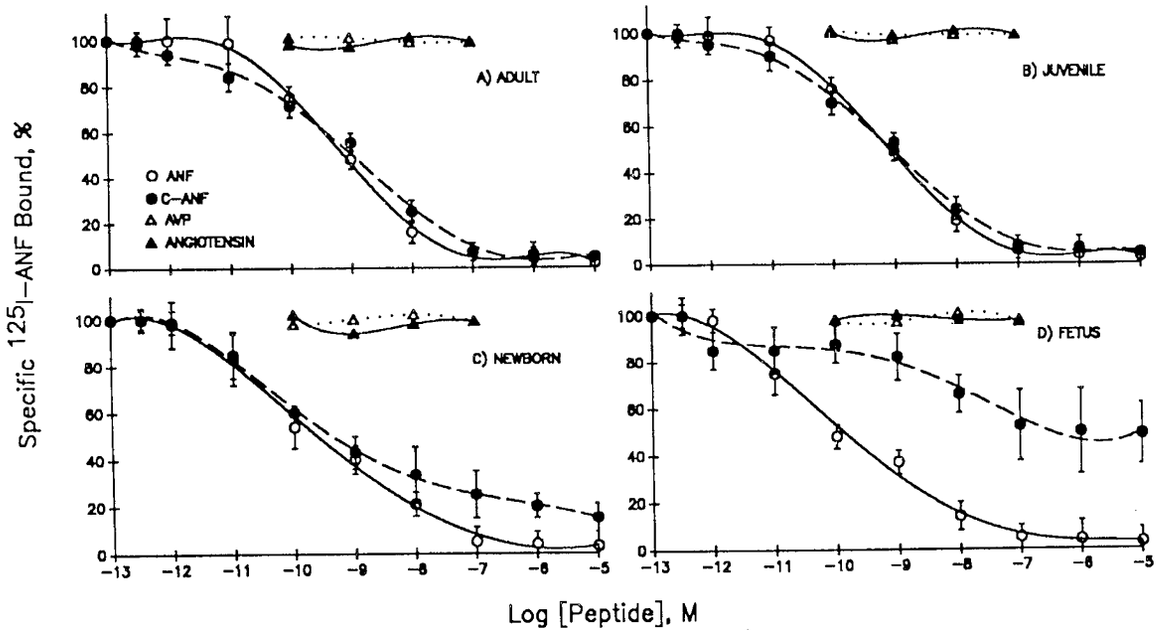

Fig. 2. Competition studies of ^{125}I-ANF binding in *A*, adult; *B*, juvenile; *C*, newborn; and *D*, fetal glomerular membranes. Values are plotted as mean ± SEM of three separate experiments.

Table 1. *Rabbit glomerular ANF Kd and B_{max} in developing and adult rabbits*

	Kd (pM)	B_{max} (fmol/mg protein)
Fetus (29 d), $n = 28$	76 ± 12	10 ± 1*
Newborn (3 d), $n = 21$	174 ± 52	12 ± 3*
Juvenile (28 d), $n = 6$	156 ± 59	30 ± 8
Adult, $n = 6$	177 ± 59	74 ± 15

* $p < 0.05$ *vs* adult values.

ovine fetal kidney is less responsive to ANF than the adult kidney, and we have reported a relatively increased renal responsiveness to ANF in the younger (114-d) ovine fetus *versus* the near-term (130-d) fetus despite similar ANF plasma levels and clearance values at these fetal ages (35). This age-dependent decrease in response to ANF in the 3rd-trimester fetal sheep suggests developmental regulation of guanylate cyclase-linked receptors in the developing mammal. It is unclear whether the decreased ANF binding capacity in fetal tissue *versus* adult tissue is due to down-regulation of uncoupled receptors or receptor occupation (30) secondary to the relatively high plasma ANF levels found in the fetus (18, 34, 35). However, nonguanylate-linked receptors ap-

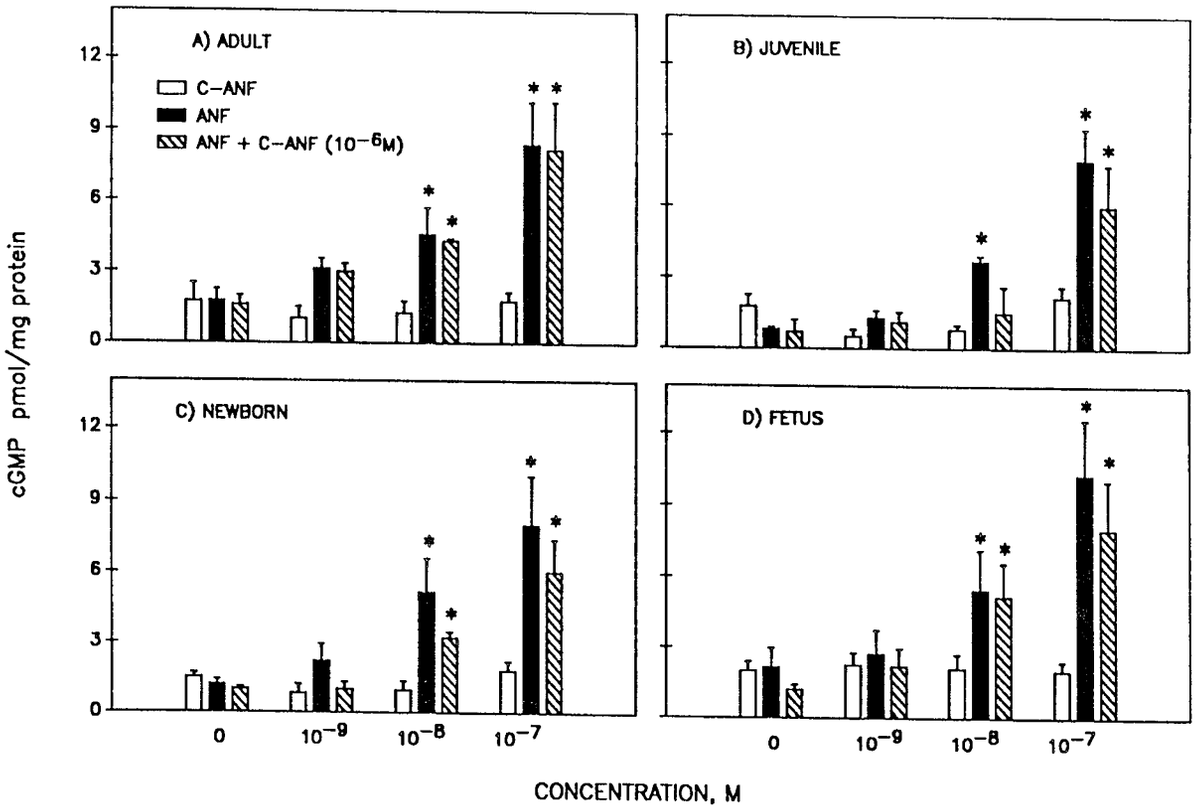

Fig. 3. Dose response of cGMP to increasing concentrations of C-ANF (*open bar*), ANF (*closed bar*), and ANF in the presence of 10^{-6} M C-ANF (*hatched bar*) in glomeruli from *A*, adult; *B*, juvenile; *C*, newborn; and *D*, fetal rabbit. Values are the mean ± SEM of three separate experiments. *, $p < 0.05$ *vs* control values.

pear to be more sensitive to down-regulation in response to volume and sodium fluctuations or prolonged exposure to high concentrations of ANF (28, 30).

The correlation of functional ANF receptors and biologic responses in developing target tissues and intact animals remains unclear. Nakazawa *et al.* (36) demonstrated that ANF decreased cardiac output and arterial pressure in the chick embryo (stage 21), and Balaraman *et al.* (37) observed no difference in ANF-induced aortic ring relaxation in guinea pig fetuses at 55–60 d gestation (term = 68 d) relative to adults. In addition, Schiffrin *et al.* (38) observed similar ANF-induced relaxation in precontracted vascular tissue in both young (4–8 wk) and older (16 wk) rats, despite a significant reduction in ANF binding sites in the younger animals. Thus, despite *in vitro* evidence for functional renal and vascular ANF receptors, *in vivo* systemic ANF infusion studies in rats (19) and sheep (20) indicate blunted renal and cardiovascular responses to ANF in immature animals when compared with adults. In addition, Chevalier *et al.* (39) observed that in young rats, ANF-induced urinary cGMP and sodium excretion do not correlate, suggesting that cGMP may not adequately reflect the renal action of ANF. Although the mechanism(s) for the differences between the *in vitro* and *in vivo* responses to ANF is not clear, ANF metabolic clearance, altered hemodynamics, and neurohumoral reflexes all may contribute in intact, immature animals. For example, despite *in vitro* evidence of a relatively decreased abundance of clearance receptors in the developing kidney, plasma ANF clearances are higher in fetal than in adult sheep (40), suggesting that additional clearance site(s) or mechanisms (*i.e.* placental metabolism) may account for the diminished fetal action of ANF (35). In addition, lower systemic perfusion pressure and decreased organ blood flow may minimize or preclude ANF-induced cardiovascular or renal effects in the immature animal.

In summary, we have demonstrated that fetal rabbit glomeruli manifest high-affinity and low-capacity ANF binding consistent with an ANF receptor. The associated dose-dependent increases in cGMP production are consistent with the presence of a guanylate cyclase-linked receptor population. Of special interest was our observation that, despite increases in glomeruli ANF receptor numbers with maturation, cGMP generation remained unchanged. The developmental changes in receptor density without differences in receptor responsiveness suggest that the enhanced renal response to ANF with maturation is not receptor-mediated or guanylate cyclase-dependent. In turn, formation or inhibition of second messengers other than cGMP through ANF receptor subtype(s) occupancy may differ with maturation. Future studies investigating the presence, characteristics, and density of ANF receptor types in developing target organs during maturation will better define the physiologic role of ANF in developing mammals.

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