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ATRIAL NATRIURETIC FACTOR AND RENAL FUNCTION DURING PREGNANCY IN THE RAT

By

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A thesis submitted to the
Faculty of Graduate Studies and Research, McGill University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

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Dedicated to my parents:
Al-Haj Adem and Asha Ejel

ABSTRACT

Using the rat as a model we tested the hypothesis that pregnancy might lead to an attenuation of the diuretic/natriuretic effects of atrial natriuretic factor (ANF) to allow physiologically required fluid expansion. Our studies revealed that this was indeed the case. Effects of ANF on fluid and sodium excretion as well as on glomerular filtration rate were significantly reduced during pregnancy. This decrease in the renal effects of ANF during pregnancy was not associated with any changes in the pharmacokinetics or renal metabolism of ANF. However, binding studies demonstrated a significant decrease in *guanylyl cyclase* (GC)-linked ANF receptors and a decrease in ANF stimulated cGMP production in glomeruli and papillae of pregnant rats. The ribonuclease protection assay and Western blot analysis revealed that pregnancy caused .a significant decrease in GC-A-linked ANF receptors in renal papillae. It is concluded that pregnancy is associated with attenuation of the renal effects of ANF and this is due to a downregulation of GC-A-linked ANF receptors which might reflect physiological adjustment to facilitate fluid/electrolyte expansion.

ABRÉGÉ

Nous avons vérifié l'hypothèse que la grossesse mène à une diminution des effets natriurétiques et diurétiques de l'ANF (facteur natriurétique auriculaire) qui résulte en une augmentation des fluides corporels. Nos recherches chez le rat ont démontré que l'action de l'ANF sur l'excrétion de l'eau et du sodium ainsi que sur la filtration glomérulaire est diminuée de manière significative pendant la grossesse. Cette diminution n'est pas associée à des changements pharmacocinétiques ou du métabolisme rénal de l'ANF. L'analyse des interactions "ligand-récepteur" a démontré une baisse des récepteurs de peptides natriurétiques associés à la guanylate cyclase (GC) du glomérule et de la papille rénale. De plus, la production de cGMP causée par l'ANF est dimimuée chez le rat femelle en gestation. Le test de protection aux ribonucléases et l'analyse par immunobuvardage ont également démontré que la grossesse diminue l'expression des récepteurs de peptides natriurétiques GC de type A (GC-A) des papilles rénales. En conclusion, la grossesse atténue l'action rénale de l'ANF en diminuant les récepteurs de peptides natriurétiques GC-A. Cet ajustement physiologique favorise ainsi l'augmentation des fluides corporels et des électrolytes pendant la grossesse.

PUBLICATIONS

1. Mulay S, **Omer S,** Vaillancourt P, D'Sylva S, Singh A, and Varma D. R. Hormonal modulation of atrial natriuretic factor receptors and effects on adrenal glomerulosa cells of female rats. Life Sci. 1994; 55: PL169-176.

2. Mulay S, Vaillancourt P, **Omer S,** and Varma D. R. Hormonal modulation of atrial natriuretic factor receptors in adrenal fasciculata cells from female rats. Can. J. Physiol. & Pharmacol. 1995; 73: 140-14.

3. **Omer S,** Mulay S, Cernacek P, and Varma D. R. Attenuation of the renal effects of atrial natriuretic factor during rat pregnancy. Am. J. Physiol. 1995; 288: F416-F422.

4. **Omer S,** Varma D.R, Cernacek P, and Mulay S. Pharmacokinetics and renal metabolism of atrial natriuretic factor during rat pregnancy. Am. J. Physiol. 1996; 271: F194-F197.

5. **Omer S,** Vaillancourt P, Peri K.G, Varma D.R, and Mulay S. Downregulation of renal atrial natriuretic factor receptors and receptor mRNA during rat pregnancy. Am. J. Physiol. 1997; 272: F87 - F93.

6. Vaillancourt P, **Omer S,** Varma D.R, and Mulay S. Downregulation of adrenal atrial natriuretic peptide receptors and receptor mRNAs during rat pregnancy. J. Endocrinol. 1997; 155: 523-530.

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1. Mulay S, **Omer S,** and Varma D. R. Inhibition of the Aldosterone suppressant, natriuretic/diuretic activity of atrial Natriuretic factor by pregnancy and progesterone in rats. 16th Annual Ontario-Quebec Investigators Meeting, Kingston, Ontario. Nov. 19-21 1992.

2. **Omer S,** Mulay S, and Varma D. R. Attenuation of the renal effects of atrial natriuretic factor during rat pregnancy. 12th International Congress of Pharmacology; Montreal, Canada. July 24-29, 1994.

3. **Omer S,** Varma D.R, and Mulay S. Downregulation of atrial natriuretic factor receptors in Renal glomeruli and papilla during rat pregnancy. FASEB Meeting; Washington, D. C. , April 14-17, 1996.

4. Vaillancourt P, **Omer S,** Varma D.R, and Mulay S. Downregulation of adrenal and renal atrial natriuretic factor (ANF) -GC receptor mRNA during rat pregnancy. 10th International Congress of Endocrinology; San Francisco, U.S.A. June 12-15 1996.

5. **Omer S,** Varma D.R, and Mulay S. Role of Nitric Oxide in Renal hyperfiltration during diabetic pregnancy in rats.(submitted) to the 79th Annual Meeting of the Endocrine Society, Minneapolis, U.S.A. June 11-14, 1997.

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ABBREVIATIONS

ADH	Antidiuretic hormone
ANF	Atrial natriuretic factor
ANG II	Angiotensin II
AVP	Arginine-vasopressin
BNP	Brain natriuretic peptide
CNP	C-type natriuretic peptide
cGMP	Guanosine 3', 5'-cyclic monophosphate
EDRF	Endothelium derived relaxing factor
ECFV	Extracellular fluid volume
GC	guanylyl cyclase
GFR	Glomerular filtration rate
IMCD	Inner medullary collecting ducts
NEP	Neutral endopeptidase
NO	Nitric oxide
OVX	Ovariectomized
PRA	plasma renin activity
PRC	plasma renin concentration

PV	Plasma volume
RIA	Radioimmunoassay
RNase	Ribonuclease
RPF	Renal plasma flow
RVR	Renal vascular resistance

CLAIM TO ORIGINALITY

To the best of my knowledge, the following are original findings some of which have been confirmed by other investigators.

1. Pregnancy leads to an attenuation of the renal effects of ANF; the effects of ANF are restored during the postpartum period.

2. The attenuation of the renal effects of ANF by pregnancy is not due to changes in its pharmacokinetics or renal metabolism.

3. Rat renal papillae primarily contain ANF GC-A receptors and almost no GC-B and ANF-C receptors.

4. The attenuation of the renal effects of ANF during pregnancy is caused by a downregulation of ANF GC-A receptors in the renal glomeruli and papillae and in turn by a decrease in ANF-induced cGMP production.

5. The modulation of the renal effects of ANF by pregnancy are partially but not completely mimicked by exogenous progesterone administration

A significant portion of data of this thesis have been reported in the following publications:

Peer-reviewed papers

1. **Omer S,** Mulay S, Cernacek P, and Varma D. R. Attenuation of the renal effects of atrial natriuretic factor during rat pregnancy. Am. J. Physiol. 288: F416-F422, 1995.

2. **Omer S,** Varma D.R, Cernacek P, and Mulay S. Pharmacokinetics and renal metabolism of atrial natriuretic factor during rat pregnancy. Am. J. Physiol. 271: F194-F197, 1996.

3. **Omer S,** Vaillancourt P, Peri K.G, Varma D.R, and Mulay S. Downregulation of renal atrial natriuretic factor receptors and receptor mRNA during rat pregnancy. Am. J. Physiol. 272: F87 - F93, 1997.

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CHAPTER ONE: LITERATURE REVIEW

1.1 INTRODUCTION

The body maintains fluid and electrolyte homeostasis through a complex interplay of regulatory mechanisms involving peptide and non peptide hormones as well as neural and neuro-humoral systems; the ultimate goal is to achieve a state of homeostasis both acutely and over a long term, despite variations in the daily input and output of fluid and electrolytes (1). Most of these regulatory mechanisms such as the renin-angiotensin-aldosterone axis, antidiuretic hormone (ADH), thirst and the sympathetic nervous system are directed towards conserving sodium and water within the body (2, 3); they influence the kidney both directly and indirectly to modify sodium and water excretion (Fig. 1.1). Moreover, various observations have implicated a counter-regulatory system(s) which is (are) responsible for increasing renal water and sodium output (4).

1.2 RENAL HEMODYNAMICS AND VOLUME HOMEOSTASIS DURING PREGNANCY

Pregnancy is accompanied by marked changes in renal hemodynamics. Gestational increase in GFR has been described in a number of species including humans, dogs, rabbits, sheep and rats. (5). Baylis and co-workers (6, 7, 8) have shown that in the conscious chronically catheterized rat, GFR starts to rise early in gestation and reaches a maximum of 30% increment above the virgin value by midterm; this increase is

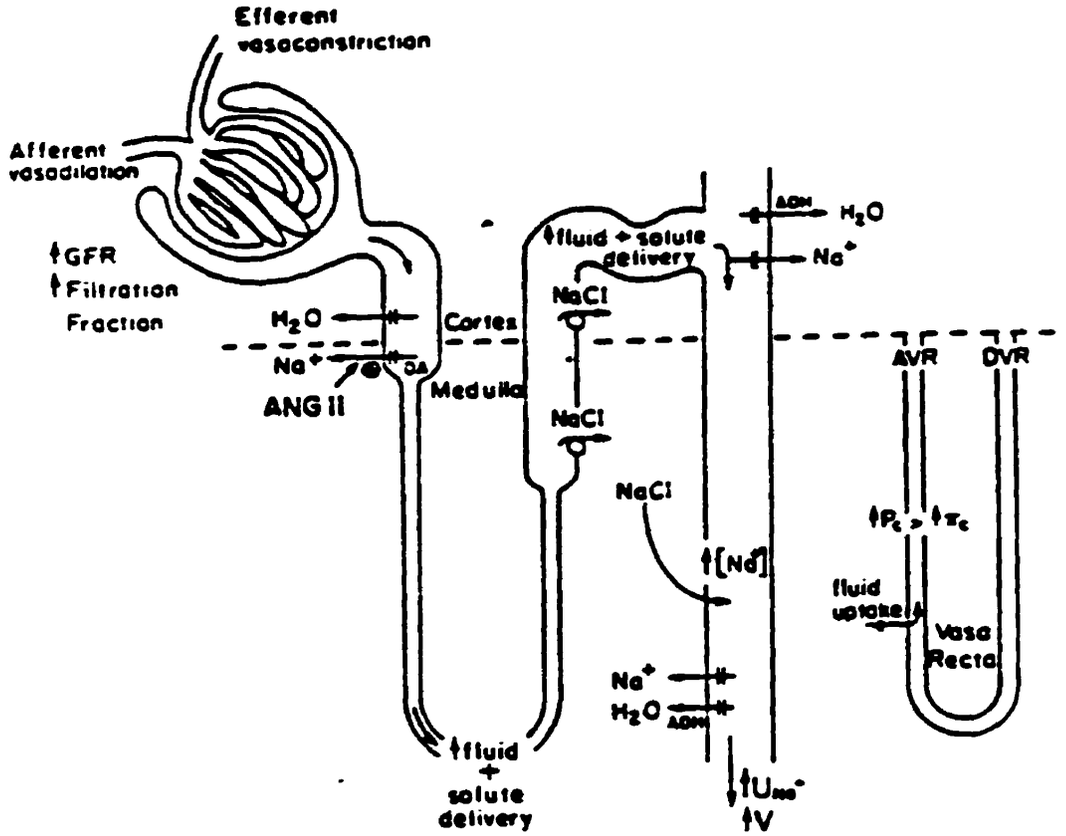


Fig. 1.1. The nephron and sites of action of different hormones that influence renal water and solute excretion.

ADH, antidiuretic hormone; ANG II, angiotensin II; AVR, ascending vasa recta; DA, dopamine; DVR, descending vasa recta; π_c, colloid osmotic pressure; P_c, vasa recta capillary hydraulic pressure (taken from ref. 119).

maintained until close to term when GFR starts to return to prepregnant values. Similar pattern of changes is also observed in pregnant women (9). The rise in GFR is accompanied by a similar rise in RPF and a fall in renal vascular resistance (RVR) (7).

Micropuncture technique has allowed workers to gain more insight into the intrarenal mechanisms that regulate GFR and its determinants during pregnancy; using this technique, Baylis *et al* (10, 11, 12) have demonstrated that the increase in a single nephron GFR and the whole kidney GFR during pregnancy is exclusively due to a rise in RPF; the other determinants of GFR, namely, filtration pressure gradient (ΔP), glomerular capillary hydrostatic pressure (P_{GC}), oncotic pressure as well as the ultrafiltration coefficient (K_f) remain unchanged at midgestation, implying that a uniform vasodilatation occurs both at the pre and post glomerular resistance vessels during pregnancy (fig. 1.2).

The mechanisms responsible for the changes in renal hemodynamics during pregnancy have yet to be delineated; it has been clearly established that the fetoplacental unit is not essential for initiating these changes. The rat readily becomes pseudopregnant (cessation of the estrous cycle and onset of dioestrus as in pregnancy) when mated with a vasectomized male and exhibits weight gain, twice daily surge in prolactin, elevated progesterone, aldosterone and renin as well as expansion of the extracellular and plasma volumes (13, 14, 15); thus, the pseudopregnant rat hormonally resembles the pregnant rat in the first 10 days of mating and exhibits all the hemodynamic changes seen in pregnancy (16, 17). Furthermore, when either progesterone or prolactin are given chronically they produce a state of pseudopregnancy, accompanied by an increase in GFR and single nephron GFR (18, 19); other workers (20) however, did not observe changes in GFR or its determinants

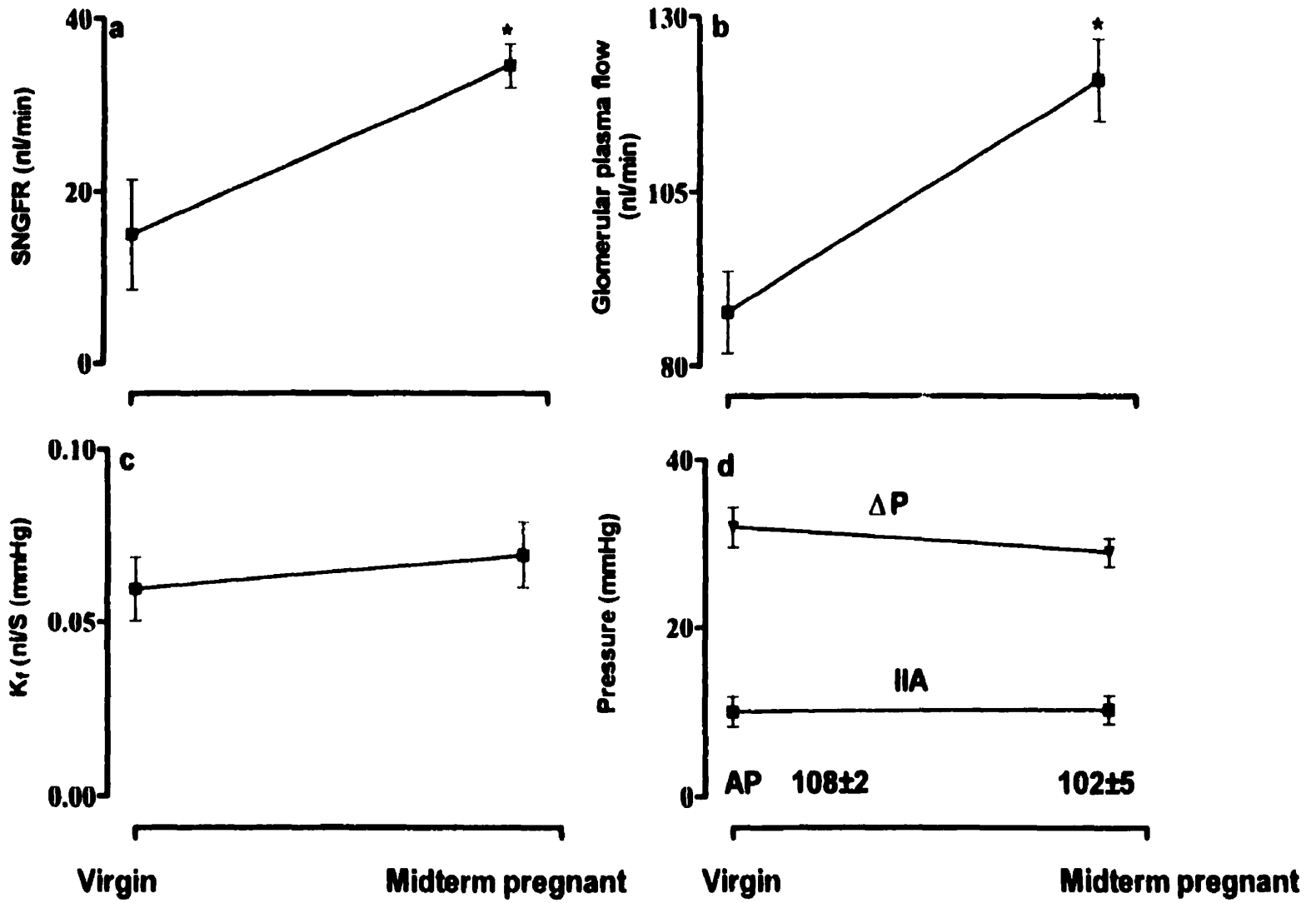

a
SNGFR (nl/min)
40
20
0
b
Glomerular plasma flow (nl/min)
130
105
80
c
K_f (nl/S (mmHg)
0.10
0.05
0.00
d
Pressure (mmHg)
40
20
0
ΔP
IIA
AP 108±2
102±5
Virgin
Midterm pregnant
Virgin
Midterm pregnant

Fig. 1.2. Changes in renal hemodynamics in virgin and midterm (12 day) pregnant Munich-Wistar rats.

a. Single nephron glomerular filtration rate (SNGFR); b. glomerular plasma flow; c. glomerular capillary ultrafiltration coefficient (K_f); d. transglomerular hydrostatic pressure difference (ΔP) and oncotic pressure at the glomerulus(π_A) and mean arterial pressure (AP). * Denotes significant difference ($P < 0.05$) from virgin value. (Taken from ref. 14).

in prolactin induced pseudopregnant rats or prolactin injected males. Despite the strong evidence that maternal factors initiate the changes in renal hemodynamics during pregnancy, the placenta is essential for maintaining these changes (21). Studies have revealed that removal of the fetoplacental unit led to decrease in GFR and RPF whereas removal of the fetuses alone did not (21).

1.3 RENAL TUBULAR FUNCTIONS DURING PREGNANCY

The increases in GFR in pregnancy are large enough to increase the filtered load of solutes which would lead to solute and volume wasting unless they are accompanied by a parallel change in tubular reabsorption. Net retention of solutes leading to an expansion of plasma and the extracellular volumes is a well-known feature of pregnancy (22). Although there is a general consensus that whole kidney water and sodium reabsorption is enhanced during pregnancy, there is controversy as to the stage of pregnancy at which this begins. Enhanced reabsorption has been reported as early as 2-3 days after mating (23), while Lindheimer *et al.* (24) did not observe changes in early gestation. However, when reabsorption was expressed as a fraction of the filtered load it was found to be increased throughout gestation in animals infused with saline (23, 24). Therefore, the general agreement is that absolute renal reabsorption is increased at some if not at all stages of pregnancy (22).

Micropuncture and lithium clearance studies (25, 26) have shown that there is increased sodium and water reabsorption in the proximal tubules during midgestation, although fractional reabsorption remained unchanged. Sodium and water are reabsorbed in parallel in the proximal tubule but this is not the case along the remaining segments of

the nephron. In the descending loop of Henle, for instance, sodium enters into whereas water leaves the nephron; sodium is reabsorbed in the ascending limb, which is virtually impermeable to water (22). Garland and Green (25, 27) have shown that less fluid reaches the distal tubule in pregnant than in virgin rats and its sodium concentration is also less in pregnant rats which would imply enhanced sodium reabsorption in the thick ascending loop of Henle. It also appears that the distal tubule and collecting ducts show increased reabsorbtion of sodium during pregnancy (22, 27), although direct proof for this is lacking.

1.4 BODY FLUID CHANGES:

Pregnancy in both humans and rodents is characterized by progressive expansion of the extracellular fluid volume (ECFV); both the interstitial and plasma volumes are increased (28). Browen *et al.* (29) have shown that in addition to the increase in ECFV, there is redistribution of this volume during pregnancy (Fig. 1.3 and 1.4); they reported that a greater proportion of the ECFV volume lies intravascularly in normal pregnant women than in nonpregnant women. In human pregnancy the increase in plasma volume can be demonstrated as early as 6 weeks of amenorrhea (30), reaching 30-40% above prepregnant values by the third trimester (31). Similar changes have also been demonstrated in the rat; using the Evan's blue dye dilution technique, small but significant increments in plasma volume were observed by day 6 of gestation in both the Sprague-Dawley and Munich-Wistar rats (16, 32); these findings are consistent with the gestational decrease in hematocrit reported by Atherton and colleagues (33) on day 6 of pregnancy. However, differences exist in the temporal profile of these changes between

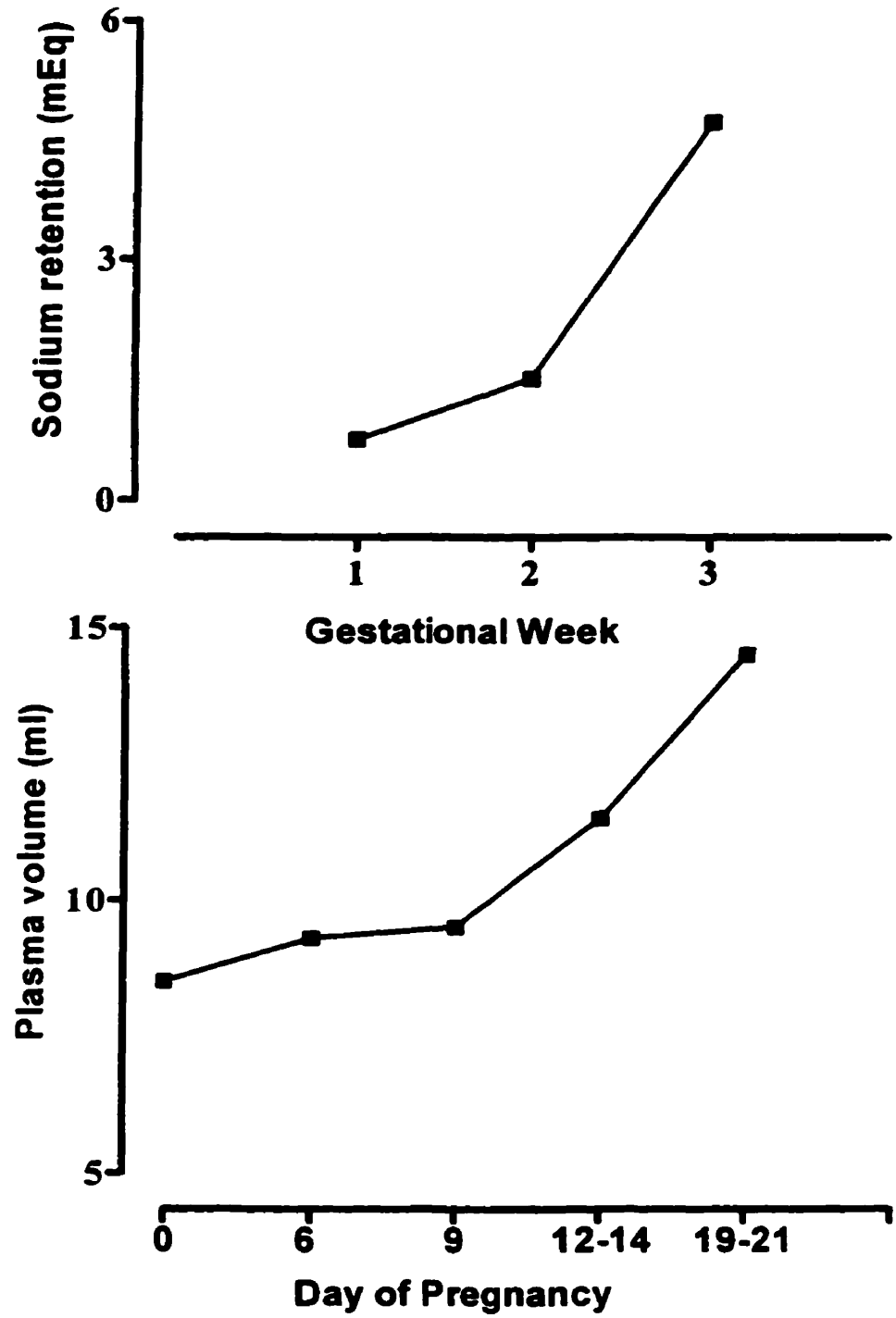


Fig. 1.3. Cumulative sodium retention (top) and serial changes in plasma volume during rat pregnancy (bottom).

(Modified from ref. 34).

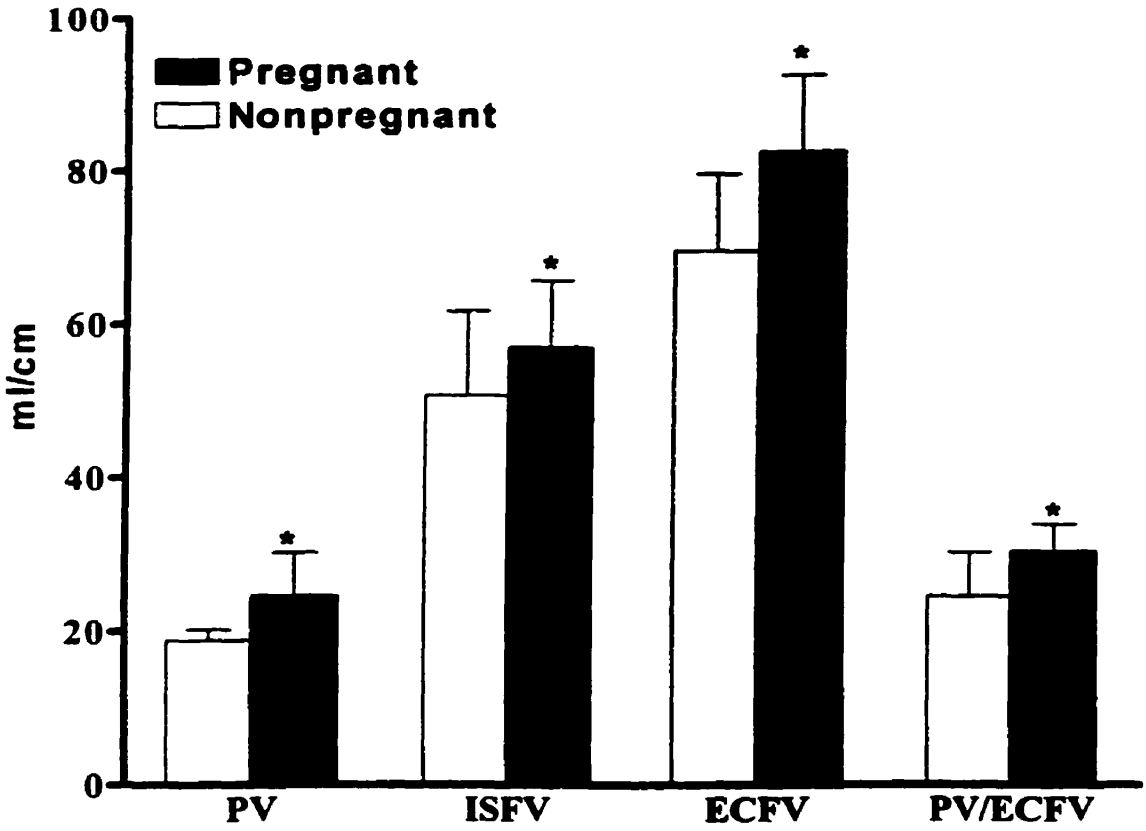


Fig. 1.4. Plasma volume (PV), interstitial fluid volume (ISFV), and total extracellular fluid volume (ECFV) in non-pregnant and pregnant women in their third trimester. Data are median and interquartile ranges.

* Significantly different from nonpregnant values (From ref. 28).

Human and rat pregnancy; maximal increments are observed in early and midgestation in humans, but 50% of the increase takes place during the final week of gestation in rats (34). The mechanisms underlying the changes in ECFV are poorly understood; however these changes are essential both to provide adequate perfusion of maternal organs and fetal beds and to ensure both maternal and fetal well being. There is a direct correlation between the degree of plasma volume expansion and fetal growth (35, 36). Pregnancies that show less than the ideal plasma volume expansion are complicated by intrauterine growth retardation (37).

1.5 VOLUME REGULATION IN PREGNANCY

As mentioned earlier, the volume of the extracellular fluid space is determined primarily by sodium, and there is a net accumulation of about 900 mmoles of sodium during human pregnancy, which represents a retention of 3-4 mmol/day (28). It was believed that this volume expansion was secondary to renal sodium retention; studies, however, have demonstrated that this might not be the case in early gestation, at least in the rat (38). Rats maintained on a zero sodium diet exhibited plasma volume expansion, which is normally observed at midgestation suggesting that the renal sodium retention was not essential for plasma volume expansion (38). However, net sodium retention appears to be critical to the massive fluid expansion that occurs later in pregnancy; in fact sodium restriction at this stage not only prevents volume expansion but also compromises pregnancy (39). Positive sodium balance has been documented in rats in the second half of gestation (16, 40).

The mechanisms responsible for this volume readjustment in pregnancy remain largely elusive and several theories have been put forward. Some workers (1, 41) consider pregnancy as an underfill state that triggers water and sodium retaining systems such as the increase in plasma renin concentration (PRC), plasma renin activity (PRA) and aldosterone; other workers (1, 41) proposed pregnancy to be an overfill state because of the increase in cardiac output, GFR and RPF.

Most workers (42, 43, 44), however, suggest that throughout pregnancy the plasma volume is sensed as normal with constant readjustment of the set point of all the regulatory mechanisms. Consistent with this notion are the changes seen in the osmoregulatory system during pregnancy; there is a resetting of the osmotic threshold for vasopressin (AVP) release and thirst to a lower point and pregnancy is associated with a significant fall in plasma osmolality of about 10 mosmol/kg H_2O despite normal plasma AVP and comparable metabolic clearance rate of AVP in the pregnant and non pregnant states (43). In addition, Baylis *et al.* (45) have studied the response of the sympathetic nervous system to moderate hemorrhage during pregnancy and found that the blood pressure and renal vasoconstrictor responses were similar in virgin, early and late pregnant rats; these observations led them to conclude that the effector mechanisms that sense volume, regulate blood pressure and modify renal vascular resistance are continually reset to recognize the progressive volume expansion as normal.

1.6 FACTORS INFLUENCING RENAL SODIUM HANDLING

1.6.1 Increased glomerular filtration rate

As described earlier both GFR and RPF increase by approximately 30-50% above nonpregnant levels which means that the kidneys receive an additional filtered load of sodium of approximately 5,000-10,000 mEq/day (46); these changes must be accompanied by a parallel increments in tubular reabsorption to prevent massive depletion of sodium. The ability of the kidney to adjust tubular reabsorption to match an increased filtered load (46), a phenomenon known as glomerulotubular balance, is not peculiar to pregnancy. It occurs whenever GFR, is acutely or chronically, elevated under a variety of maneuvers. In pregnancy, the adaptive tubular changes not only equal the large increase in the filtered load but a few additional milliequivalents of sodium are reabsorbed daily for fetal and maternal stores; these tubular adjustments to compensate for the increased filtered load during gestation have been documented in micropuncture studies in the pregnant rat (7, 47). Furthermore, the length of the proximal tubule increases early in pregnancy and by the end of the first week it is 20-25% longer than that of virgin control rats (22, 48). This increase in length is associated with increased surface area available for reabsorption. It is also significant that during rodent gestation the increase in the filtered load of solute is paralleled by proportionate rise in renal microsomal Na^+- K^+ ATPase, suggesting that this enzyme plays a role in the enhanced sodium reabsorption during pregnancy (24).

1.6.2 The renin- angiotensin-aldosterone axis

The renin-angiotensin-aldosterone axis is a major volume sensing and control system. Under normal conditions expansion of the ECFV is associated with suppression of renin release and hence a fall in plasma angiotensin II (ANG II) and aldosterone levels. This in return leads to increased renal sodium excretion and the restoration of plasma volume to normal. Conversely, reduction in ECFV is accompanied by an elevation in ANG II and aldosterone with consequent sodium retention (1, 14).

The activity of the renin-angiotensin system is influenced by the sodium, water and potassium content of the fluid delivered to the renal tubules. Sodium or fluid depletion sensed by the macula densa of the juxtaglomerular apparatus leads to stimulation of renin release; renin then acts on angiotensinogen, an α_2-globulin produced by the liver, to form angiotensin I (ANG I) (2). Angiotensin converting enzyme which is abundant in the lungs, kidney, liver and the vascular endothelium (2) converts angiotensin I to ANG II. ANG II through its interaction with specific cell surface receptors increases aldosterone secretion, produces central and peripheral vasoconstriction, renal sodium reabsorption and causes feedback inhibition of renin release (2). These effects of ANG II are mediated by AT_1 receptors (49, 50) which belong to the family of seven transmembrane domain G-protein-coupled receptors. Binding of ANG II to its receptors activates phospholipase C (PLC) (51, 52) which then causes the hydrolysis of phosphatidylinositol and the generation of 1,2-diacylglycerol (DAG) and 1,4,5-inositoltriphosphate (IP_3). IP_3 mobilizes calcium from intracellular stores (53), while

DAG activates protein kinase C (PKC) (54); the combined actions of the two leads to increased aldosterone secretion (55).

Aldosterone is synthesized in the outer zone of the adrenal cortex, the zona glomerulosa; it increases reabsorption of sodium and excretion of potassium and hydrogen by the renal collecting ducts (56). Other factors can also increase aldosterone secretion. Increase in extracellular potassium stimulates aldosterone secretion by altering the membrane potential of the glomerulosa cells (57, 58) and activating voltage dependent calcium channels. Adrenocorticotrophic hormone (ACTH) also stimulates aldosterone synthesis through a pathway that involves the generation of cAMP which activates protein kinase A (59, 60).

ANF plays an important role in inhibiting aldosterone release; the exact signal transduction pathways by which it achieves this are not fully understood. As will be described in later sections, cGMP mediates the biological activity of ANF; however, increasing intracellular cGMP, using membrane permeable cGMP analog such as 8-bromo-cGMP did not result in inhibition of aldosterone secretion (61). Some workers (62) have suggested that the action of ANF on aldosterone secretion is mediated by inhibiting adenylate cyclase via an inhibitory guanine nucleotide binding protein (G_i). However, manipulation of the adenylate cyclase system with forskolin, a potent stimulator of the catalytic subunit of adenylate cyclase or with pertussis toxin which ADP-ribosylates the α-subunit of G_i, did not prevent ANF from inhibiting aldosterone secretion (61, 63).

In both human and rat gestation, levels of renin, ANG II and aldosterone increase despite an expanded maternal volume (13, 64, 65). Whether the changes in the renin-ANG II-aldosterone axis result from or cause the changes in volume homeostasis is not clear. The production of renin and its substrate is markedly elevated during pregnancy (66). The former arises mainly from the maternal kidney while the latter arises from maternal liver (67). The fact that women on oral contraceptives or receiving estrogen replacement therapy have increased levels of renin substrate (68) led to the suggestion that placental estrogens stimulate the synthesis of renin substrate by the liver during pregnancy. The increase in renin and renin substrate during gestation invariably leads to an in increase in plasma renin activity and ANG II and thus the high circulating aldosterone levels (66). Others (69, 70) have hypothesized that the increased renin secretion is secondary to high levels of vasodilator prostaglandins during pregnancy and that the generalized vasodilatation leads to compensatory stimulation of the renin-ANG II-aldosterone system.

Despite the high circulating levels of the renin-ANG II-aldosterone, this system still responds appropriately to a variety of stimuli such as alterations in salt and water intake (71), diuretic therapy (72), saline infusion and upright posture (73, 74). Therefore, whether the changes seen in the renin-ANG II-aldosterone-axis during pregnancy result from or cause the changes in volume homeostasis is not entirely clear. Some authorities have suggested a new set point for the renin-ANG II-aldosterone-axis during pregnancy (14, 28). It is of interest that the high levels of ANG II is associated with a refractoriness to its pressor effects (75), and a downregulation of its receptors at least in platelets (76); this would permit the vasodilatation seen in pregnancy.

1.6.3 Antidiuretic hormone (ADH)

The primary actions of ADH include: an increase in water permeability of certain nephron segments (77), increase in urea permeability of the medullary collecting ducts (78) and an increase in sodium reabsorption in the thick ascending loop of Henle (79). In addition, some workers (80) have reported a natriuretic effect of infused ADH in the rat, while others (81, 82) could not demonstrate this effect. Plasma concentration of ADH remains unchanged in both the human and rat pregnancy (83). However, there are conflicting reports on its role in sodium excretion during pregnancy; both a natriuretic (84) and antinatriuretic (85) effects have been reported.

There are profound changes in the osmoregulatory system during pregnancy (44). Normally, plasma osmolality ranges between 280-300 mosmol/kg H_2O; its maintenance within this narrow range is due to a tight balance between water excretion determined by the action of ADH on the collecting ducts and water intake governed by the thirst mechanism (86, 87). During pregnancy plasma osmolality falls by 8-10 mosmol/kg H_2O (88); this fall starts soon after conception and becomes significant by gestational week five in women. If a decrease in plasma tonicity of similar magnitude to that seen in pregnancy occurs in nonpregnant individuals, ADH release would be suppressed and prompt diuresis would ensue (44, 89). However, this does not happen in pregnancy and both pregnant women and rats have normal plasma ADH and are able to maintain their new plasma osmolality within a narrow range responding to fluid loading or restriction by appropriate dilution and concentration of the urine (44). Durr and colleagues (83) have shown that despite the marked decrease in plasma osmolality during rat pregnancy, basal

plasma ADH levels and urine osmolality were similar in gravid and virgin Sprague-Dawley rats. Following oral water loading both pregnant and nonpregnant animals suppressed ADH to undetectable levels and their urine became maximally diluted. These observations led to the conclusion that the osmotic threshold for ADH release was lowered during pregnancy. Koehler *et al.* (89) have shown that the osmotic threshold for ADH release normalizes by the sixth day of lactation in rats.

1.6.4 Nitric Oxide (NO)

NO is a potent vasodilator which is produced by vascular endothelium and other tissues (89a). In 1980 Furchgott and Zawadzki (89b) demonstrated that the vascular relaxation induced by acetylcholine was dependent on the presence of the endothelium and provided evidence that this effect was mediated by a labile humoral factor, later known as endothelium derived relaxing factor (EDRF). Subsequent work identified EDRF as NO; it is synthesized from the amino acid L-arginine (89a, 89c) by the enzyme nitric oxide synthase (NOS). NO stimulates cytosolic *guanylyl cyclase* leading to generation of cGMP which is considered to be its second messenger (89a). This is in contrast to ANF which stimulates particulate *guanylyl cyclase*. NO has been implicated in a number of pathophysiological states including pre-eclampsia (89a).

Recently, studies have implicated nitric oxide (NO) as an important factor responsible for the vasodilatation seen in pregnancy. It has been shown that NO synthesis is increased in both pregnant and pseudopregnant rats and acute administration of NO synthesis blockers such as L-nitro-arginine-methyl-ester (L-NAME) restores both GFR and blood pressure to prepregnant levels (90, 91). It is also known that plasma and

urinary levels of cGMP (second messenger for NO) are increased in pregnant women and pregnant and pseudopregnant rats (92, 93); this is believed to reflect increased tissue production as the metabolic clearance of cGMP remains unaltered during pregnancy (93). In addition, Baylis and Angles (94) have reported increased plasma and urinary levels of the stable NO products NO^-_2/NO^-_3 during pregnancy in rats; chronic NO inhibition in conscious pregnant rats suppressed the midterm renal vasodilatation, plasma volume expansion and the fall in blood pressure; infact, these rats developed hypertension and showed poor maternal and fetal outcome compared to control animals (94). Thus, collectively these studies show that NO plays an important role in the adaptations associated with pregnancy.

1.6.5 Progesterone and estrogen

Pregnancy is characterized by gradual increase in plasma progesterone, which rapidly declines just before parturition (13). Estrogen also increase but a significant increase in rats is observed from day 10 onwards (13). It is thus likely that these hormones, among other factors, also play a role in volume homeostasis during pregnancy.

Progesterone: Progesterone produces a natriuretic effect in humans (95) and inhibits the sodium-retaining effect of exogenous mineralocorticoids in adrenalectomized animals (95, 96). However, other investigators (18, 97) did not observe a natriuretic action following administration of progesterone into rats, although increased potassium retention did occur. Thus, it is not clear whether progesterone might play a direct role in renal sodium handling during pregnancy.

Estrogens: Chronic estrogen treatment is associated with sodium retention in humans (98); its effect on sodium and water retention in the rat is not clear. While some workers (99) reported a decrease in sodium excretion in estrogen-treated rats, others (13) did not observe any effect. Moreover, the significance of the role of estrogen in the renal changes that accompany pregnancy in the rat is further complicated by the fact that estradiol levels do not increase significantly until mid pregnancy when they show a rapid rise until parturition occurs (13, 100). Thus, it seems unlikely that estrogen contributes to salt and water retention in early gestation.

1.6.6 Prolactin:

This hormone is involved in volume and osmolality homeostasis in amphibians, birds and fish (101); its role in mammals however is controversial. It has been reported (102, 103) that acute administration of prolactin to conscious as well as anesthetized rats reduced sodium and water excretion; other workers (104, 105), however, observed a reduced water and sodium excretion following chronic but not acute treatment with prolactin. Changes in sodium and water reabsorption were detected in ovariectomized rats (105), and in hyperprolactinemic males (106); these effects might be due to a direct action of prolactin on proximal tubular reabsorption (19). The observations that prolactin levels rise early in gestation (107) led to suggestions that it might account for some of the renal functional changes in the pregnant rat (19). However, since prolactin can act synergistically with angiotensin as dipsogen (108), its actions could be due to alterations in ECFV, rather than a direct action on kidney function.

1.7 NATRIURETIC PEPTIDES:

There is considerable evidence that natriuretic peptides play a role in fluid and electrolyte homeostasis during pregnancy (4). Since this thesis principally involves atrial natriuretic factor and pregnancy, these peptides are reviewed below in a greater detail.

The possibility that the heart and in particular the atrium might be an endocrine organ involved in the regulation of extracellular fluid volume (ECFV) was suspected for a long time (109). It was observed decades ago that, the distention of the atrium was accompanied by an in increase in urine flow and it caused an inhibition of circulating levels of renin in anesthetized dogs (110, 111, 112, 113). Furthermore, "very small bodies" were observed on electron microscopic examinations of guinea-pig atrial myocytes (114); Jamieson and Palade (115) provided a more detailed description of these granules, which were found in mammalian (including human) atria but not in the ventricles. Subsequent work showed that experimentally induced disturbances in water and sodium balance altered atrial granularity; this lead de Bold to suggest 'a relationship between atrial specific granules' and the regulation of water and electrolyte balance (116, 117). A milestone in this regard, was the discovery by de Bold *et al.* (118) in 1981 who demonstrated that the injection of atrial extracts into rats induced prompt and vigorous diuresis and natriuresis and a fall in blood pressure. Subsequent research lead to the identification and characterization of a new family of peptides known as the natriuretic peptides.

The natriuretic peptides through their action on the vasculature, kidney and

adrenals serve to lower systemic blood pressure and reduce intravascular volume (119). The decrease in blood pressure is the result of reduced peripheral vascular resistance, diminished cardiac output and decreased intravascular volume (119). Natriuretic peptides act on specific receptors in the kidney to increase glomerular filtration rate (GFR), inhibit sodium transport and suppress renin release; these combined effects are responsible for diuresis, natriuresis as well as a reduction of arterial blood pressure (119, 120). The natriuretic peptides also act to lower blood pressure and intravascular volume by inhibiting aldosterone synthesis both directly through their action on adrenal zona glomerulosa cells and indirectly by suppressing renin release from the renal juxtaglomerular apparatus (119).

1.8 THE FAMILY OF NATRIURETIC PEPTIDES:

To date four natriuretic peptides have been characterized and their sequence identified (fig. 1.5); these are atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP) and urodilatin. All of these natriuretic peptides have a 17 amino acid ring structure that is essential for biological activity and some of the amino acid residues are conserved across the family (119, 121).

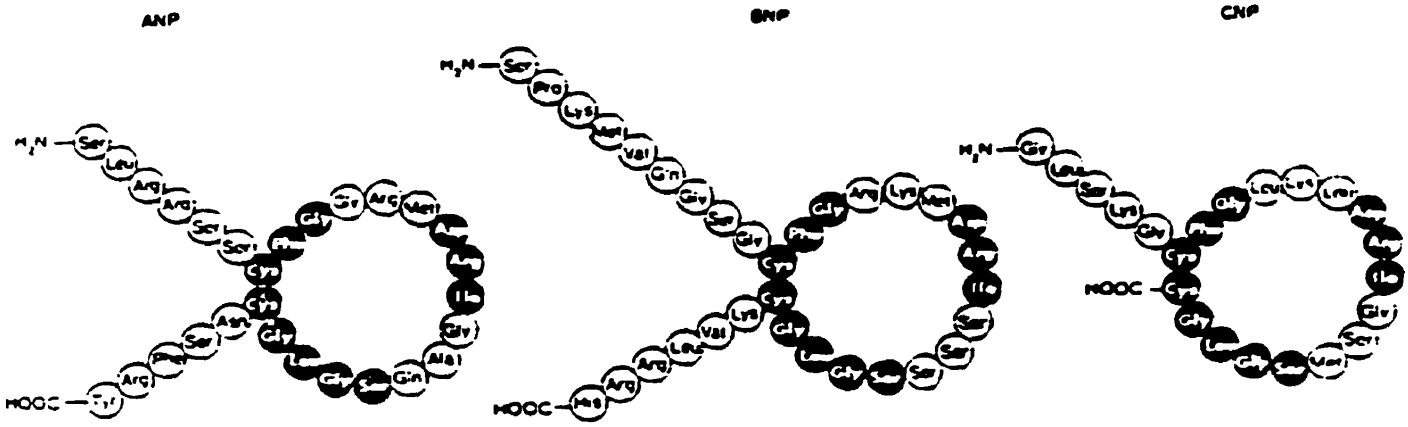


Fig. 1.5. Structure of human natriuretic peptide family:
Atrial natriuretic factor (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). Filled circles are amino acids that are common to the three members of the human natriuretic peptide family. Urodilatin (not shown) is identical to ANF except for four additional amino acids (Thr-Ala-Pro-Arg) at the amino terminal. (Taken from ref. 121).

1.8.1 Atrial natriuretic factor (ANF):

ANF, the first natriuretic peptide to be isolated and identified, is present in high concentrations in atrial tissues of many species (119, 122, 123). The biologically active form of ANF is a 28 amino acid peptide with a 17 amino acid ring structure closed by a disulfide bond between two cysteine residues (124); its amino acid sequence is highly conserved across species such that all mammals having an identical sequence except for a single amino acid substitution. Thus , humans, porcine, canine, bovine and ovine ANF have a methionine residues at position 110, while rabbits, rats and mice have an isoleucine residue, instead of methionine (119, 125, 126).

A single ANF gene has been identified in all mammalian species examined to date (127, 128, 129) and there is a close homology in the structural organization and nucleotide sequence of the ANF gene from various species (127, 128, 129). The human ANF gene transcribes an mRNA that encodes 151 amino acid precursor, prepro-ANF. The ANF gene contains three exons and two intervening introns with regulatory elements upstream (5') of the coding sequence. Exon I encodes the leader signal peptide and the first 20 amino acids of pro-ANF; exon II encodes the bulk of pro-ANF peptide except for the last residue which is encoded by exon III (127, 130). Immediately upstream from the transcription initiation site, is a TATAAA sequence (homologous to consensus promoter sequence for RNA transcription) preceded by a region containing regulatory elements (127, 131). These regulatory elements are located 500 kb or 2.4 kb upstream from the transcription initiation site in humans and rats, respectively, and may be involved in tissues specific expression of the gene (131, 132). An AP1-like binding site which may

bind the products of c-fos and c-jun, is among the regulatory elements identified in both the human and rat genes (133). In addition, the gene also contains a cAMP response element and a region required for glucocorticoid responsiveness (134).

As is the case with all secreted peptides, prepro-ANF contains a 25 amino acid leader sequence essential for translocation of the nascent peptide from the ribosome to the rough endoplasmic reticulum; subsequent cleavage of the leader sequence gives rise to pro-ANF (119).

Several factors regulate ANF gene expression; water and sodium deprivation decrease ANF mRNA by 30-70% (135), whereas administration of the mineralocorticoid, deoxycorticosterone acetate (DOCA) to adrenalectomized animals results in an increase in ANF mRNA by 70% (136). The direct mediators of these *in vivo* effects are not well understood but changes in atrial stretch associated with volume shifts may play an important role in these models. Also, glucocorticoid and thyroid hormones increase ANF mRNA, both in intact animal models as well as in cultured cardiomyocytes (137); these findings are consistent with the presence of the glucocorticoid receptor binding consensus response element that has been identified in the second intron of the human gene (134). Calcium has also been implicated in the regulation of ANF gene expression; it has been shown that extracellular calcium (137) increases ANF mRNA by 2-3 fold while a decrease in calcium reduces ANF transcripts. Calcium plays an important role in the secretion and release process of many secretory proteins, and atrial stretch the major stimulus for ANF release, is calcium-dependent (138, 140). Thus calcium not only increases ANF gene transcription but also the release of ANF (139, 140).

It is noteworthy that, during fetal life the main source of ANF are the ventricles, whereas ANF synthesis switches to atrial tissues soon after birth (141, 142). Conditions that increase left ventricular wall stress such as ventricular hypertrophy, cardiomyopathy, long standing hypertension and congestive heart failure lead to increased ANF gene expression by the ventricles (143, 144). The mechanisms underlying these developmental and pathophysiological shifts in ANF gene expression between the ventricles and atria have not been clearly delineated, but are probably related to the hemodynamic alterations associated with these conditions.

1.8.2 ANF processing and release:

cDNA analysis of ANF predict a 151 amino acid peptide which is initially translated as 151 amino acid residue prepro-ANF (145). Removal of the 25 amino acid from the amino terminal by signal peptidase yields ANF_{1-126} or pro-ANF (145, 146, 147). The first post translational modification is the formation of an interchain disulfide bond between cysteine 105 and 121 to form the 17 amino acid ring that is essential for the biological activity of ANF(148, 149); another post translational event is the trimming of two arginine residues at position 127 and 128 by carboxy peptidase E (150), an enzyme known to remove basic amino acid residues from the C-terminal of many peptide hormone precursors. However, the most important modification is the formation of ANF_{99-126} (also designated as ANF_{1-28}), from ANF_{1-126} or pro-ANF. ANF_{1-28} is the major circulating bioactive form of the peptide (Fig. 1.6).

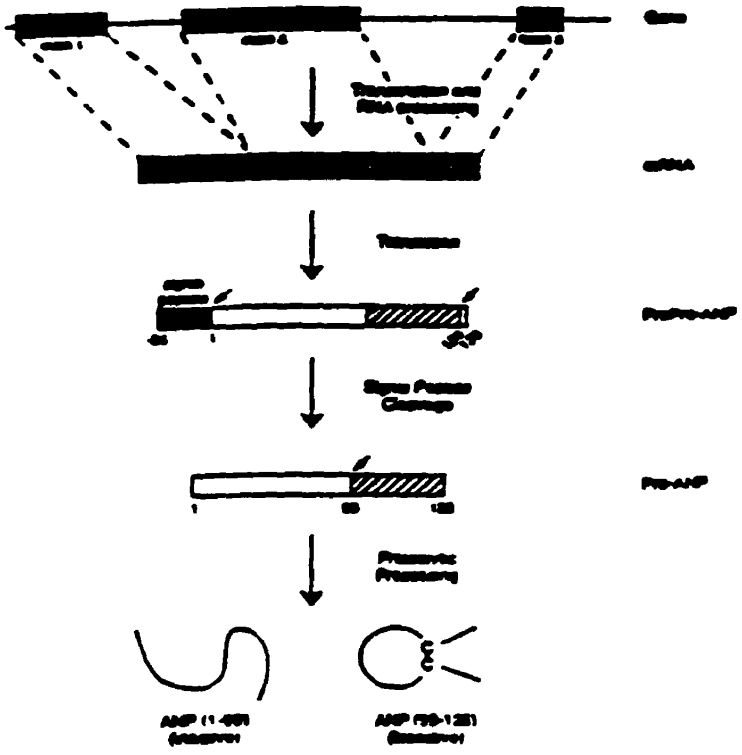


Fig. 1.6. Schematic diagram of the synthesis of atrial natriuretic peptide. (Taken from ref. 210).

Despite the wealth of information available on ANF, the enzyme responsible for cleaving ANF_{99-126} from ANF_{1-126}, its cellular location and the exact site of formation of ANF_{99-126} is still uncertain (119). However, most workers agree that this cleavage occurs during or soon after the secretion from atrial myocytes. It is evident that the enzyme responsible for the cleavage of pro-ANF does not circulate in plasma as studies from isolated perfused hearts demonstrated that plasma components were not required to release ANF_{99-126} (149, 150). Similarly, cultured atrial cells maintained in a serum free-medium accumulated pro-ANF but released ANF_{99-126} only when the glucocorticoid dexamethasone was added to the culture medium (150, 151). Moreover, atrial cells cultured in the presence of cytosine arabinoside (which inhibits the growth of all cell types except atrial myocytes) were able to release ANF into the culture medium. Collectively these and other observations lead to the conclusion that the final ANF post-translational event takes place within the heart, probably following its secretion, on the surface of the myocyte and that the enzyme responsible for this process is glucocorticoid-inducible (151, 152).

The major stimulus for ANF release is atrial stretch or distention produced by increased atrial filling pressure due to an increase in intravascular volume (139). Physiological manipulations which acutely increase circulating ANF such as volume expansion, water immersion and vasoconstrictor substances also increase atrial wall tension. Besides atrial stretch, a variety of humeral factors have been identified as ANF secretagogues; these include among others: α_1-adrenergic agonists, glucocorticoids, endothelin and calcium (150, 151, 153, 155). However, the cellular events and processes that translate mechanical stretch into ANF release are yet not exactly clear (131).

Protein kinase C (PKC) and calcium are considered to be most important signaling mechanisms for the of ANF (155, 156). Various studies have shown that stimulation of PKC leads to several fold increase in ANF release from cardiomyocytes while inhibitors of PKC tended to inhibit ANF release (157). An important mechanism in cellular physiology is the generation of inositol triphosphate (IP_3) and diacyl glycerol (DAG) by the action of phospholipase C (PLC) on phosphoinositides. IP_3 then stimulates the release of sequestered calcium from intracellular organelles to the cytoplasmic compartment while DAG activates PKC; IP_3 and DAG act synergistically to regulate the function of many endocrine organs (158). Activators of PKC such as 12-O-tetradeconyl phorbol 13 acetate (TPA) and phorbol 12,13-dideconate (PDD) which are structural analog of DAG caused several fold increase in ANF release both in isolated perfused heart preparations as well as in cultured myocytes (156, 157); on the other hand, 4α-phorbol 12, 13-dideconate, a phorbol ester analog that does not bind PKC, was inactive as ANF secretagogue (156). It has also been demonstrated that activators of PLC and phosphoinositide hydrolysis increase ANF release in a manner similar to that of phorbol ester activation.

It is of interest that α_1-adrenergic agonists such as phenylephrin stimulate ANF release in different experimental models including isolated perfused hearts, isolated atria and cultured myocardial cells (153, 154, 159); this can be blocked by the calmodulin antagonist W-7 , the PKC inhibitor H-7 as well as by selective α_1 -adrenergic blockers (160). Another potent ANF secretagogue is endothelin which has been shown to stimulate ANF release under various experimental settings as mentioned for phenylephrin. On a molar basis, endothelin is the most potent ANF secretagogue (154, 161). It is noteworthy

that both endothelin and α_1 -adrenergic agonists activate PLC and PKC. On the other hand, activators of protein kinase A (PKA) have been shown to inhibit ANF release (156).

It is well established that calcium is involved in the secretion and release of many hormones. However, the role of extracellular and intracellular calcium in ANF release varies with the experimental conditions (156). For instance, the calcium ionophor A23187 increased ANF secretion in isolated spontaneously beating rat hearts and neonatal cultured atrial myocytes; this effect was inhibited by calcium blockers nifedipine and verapamil (162, 163). In quiescent cardiomyocytes, atria or hearts neither an influx of calcium nor the release of intracellular calcium was required for constant ANF release (163).

1.8.3 Brain natriuretic peptide (BNP):

Sudoh *et al.* (164) reported in 1988 the discovery of a peptide in porcine brain that exhibited functional and structural homology to ANF; it is the same peptide that was identified from rat ventricles as iso-ANF by Flynn *et al.* (165). Although Sudoh *et al* called this peptide BNP due to its isolation from brain tissues, subsequent work revealed that BNP secretion was not confined to the brain. Infact, the heart was the main source, although it is synthesized by other tissues (166,167).

The structural organization of the BNP gene is similar to that of ANF (168, 169, 170); it consists of three exons with two intervening introns; like ANF, the second exon encodes the bulk of the peptide. A region downstream from the coding sequence contains an ATTTA motif which is believed to confer instability to mRNA and contributes to the

shorter half-life to BNP mRNA in the cell; this sequence is not present in the ANF gene (170).

The human BNP gene encodes a 134 amino acid prepro-BNP; removal of a 26 amino acid signal peptide yields a peptide of 108 amino acid residue, pro-BNP (171, 172); further processing gives rise to the mature, biologically active 32 amino acid BNP-32.

BNP retains the 17 amino acid ring structure of the natriuretic peptide family. However, in contrast to only one amino acid difference between species within the ANF ring, there is only about 59% identity between equivalent positions in BNP (131). Infact, there is greater homology between ANF and BNP within species than there is between BNP molecules from different species (131).

Immunocytochemical and other studies show that BNP is co-localized with ANF in atrial granules and ventricles; there is a 100-fold higher concentration of BNP in the atria than in the ventricles (173, 174). Furthermore, ANF is the dominant hormone with BNP being 2-5% and 20-40% of that of ANF in the atria and the ventricles, respectively (121, 166, 174). Perfusion studies with rat hearts show that, 60% of BNP secretion was maintained after removal of the atrium, but ANF release was less than 5% (174), indicating that ANF is secreted mainly from the atria while most BNP originated from the ventricles; consistent with this are the marked elevations in plasma BNP in certain pathological conditions such as ventricular hypertrophy, myocardial infarction and congestive heart failure, where it is known that ventricles release large quantities of ANF

as well. These observations have lead to the suggestion that BNP could serve as a marker of ventricular dysfunction (174).

1.8.4 C-Type natriuretic peptide (CNP):

Sudoh *et al.* (175) in 1990, reported the identification of a new member of the natriuretic peptide family from porcine brain, which was designated CNP. The concentration of CNP in the brain far exceeds that of ANF and BNP (176); it is also produced by endothelial cells and the heart (177). The precursor forms of CNP are highly conserved. For instance, there is 97% and 96% homology between the rat and pig, and human and pig precursors, respectively, making CNP precursors among the most highly conserved of the natriuretic peptide family (178, 179).

The CNP gene contains two exons and one intron; the region upstream exon I in both humans and pig contains cis-regulatory elements including TATAAA sequence, two GC-boxes and an inverted CCAAT (179,); these latter sequences are neither present in the ANF nor in the BNP genes, suggesting different regulation of the three genes. The human genomic sequence of CNP gene predicts a 126 amino acid prepro-CNP that is cleaved to 103 amino acid pro-CNP similar to that of rat and pig gene products (180). The biologically active forms of CNP (CNP-22 and CNP-53) are contained within the carboxy-terminal of pro-CNP. CNP-53 is cleaved from pro-CNP, it is not clear, however, whether CNP-22 is cleaved directly from pro-CNP or CNP_{53}.

CNP is thought to function as a neurotransmitter and as a paracrine hormone (121); it induces vascular smooth muscle relaxation and exhibits antiprolifrative properties following vascular injury (181, 182).

1.8.5 Urodilatin or renal natriuretic peptide (RNP):

The kidney is not only one of the major target organs of action for the natriuretic peptide family (119), it also elaborates a natriuretic peptide that closely resembles ANF (183). RNP was isolated from human urine by Schultz-Knappe *et al.* (184) in 1988, and was named urodilatin. Amino acid and sequence analysis revealed a peptide identical to human α-atrial natriuretic factor (αhANF) but with an additional four amino acids at its NH_2-terminal. Schultz-Knappe *et al.* (184) proposed that the peptide was produced within the kidney from the ANF gene but with different post-translational processing, yielding the 32 amino acid peptide. Thereafter, Gagelmann *et al.* (185) incubated both urodilatin and ANF with cortical membrane preparations from dog kidney and demonstrated that urodilatin was resistant to the action of peptidases which degraded ANF; this observation meant that, unlike ANF, urodilatin is quite resistant to the action of endopeptidases that are present in abundance in the brush border of the renal proximal tubules (186, 187). These endopeptidases called neutral endopeptidase or encephlinases are known to degrade many peptides one of which is ANF.

Further evidence that the kidneys synthesize a natriuretic peptide came from the work of Ritter *et al.* (188), who demonstrated using an enzyme immunoassay for cardiac ANF, the existence of a peptide similar or identical to ANF in the media of renal cell cultures as well as in extracts from these cells. In addition, when renal cells were incubated overnight with radiolabeled methionine, labeled ANF-like immunoreactivity was detected within the media, suggesting that the ANF- like immunoreactivity was synthesized *de novo* in the renal cells; consistent with these findings are

immunohistochemical and other studies that localized ANF in renal tubules (189). Although the above evidence is very compelling for a renal origin of a natriuretic peptide, conclusive data from northern blot analysis or *in situ* hybridization demonstrating the localization of urodilatin mRNA within the kidney is still lacking (183).

Urodilatin mimics the effects of ANF both *in vivo* and *in vitro* (183). Injection of urodilatin intravenously or directly into the renal artery produced prompt diuresis and natriuresis. Urodilatin has been reported to be more potent than ANF as a diuretic and natriuretic agent by some but not all investigators (190, 191). Urodilatin is similar to ANF in its binding characteristics and in its ability to stimulate cGMP although some workers found it to be less potent in stimulating *guanylyl cyclase* coupled ANF receptors (192).

There is a debate regarding the role of ANF and urodilatin in sodium and water regulation. The fact that urodilatin possesses more potent diuretic and natriuretic and less vasorelaxant activity than ANF, together with the fact that it is synthesized within the kidney and is absent from plasma lead some investigators to question the physiological role of ANF in fluid and electrolyte homeostasis (191). This notion was further supported by the inconsistencies in the diuretic and natriuretic effects of ANF under various experimental settings (183, 193). Thus, some (193, 194) have suggested that the role of ANF is more as a vasoprotective agent rather than a sodium regulator whereas urodilatin plays an important role in regulating renal sodium excretion. The role of ANF regulating sodium balance was even more challenged by the findings in transgenic mice, which remained in sodium balance despite several fold elevations in plasma ANF and the only persistent change in these transgenic animals was a low blood pressure (194).

1.9 MECHANISM OF ACTION OF NATRIURETIC PEPTIDES:

Similar to other peptide hormones, natriuretic peptides interact with specific cell surface receptors in order to evoke physiological responses; this ligand receptor interaction in turn leads to the generation of cGMP (119). cGMP then activates various intracellular events such as activation of cGMP dependent protein kinase and lowering of intracellular calcium; these processes eventually lead to the expression of the physiological actions of ANF and related peptides (119). In addition, ANF may affect target cells by modulating adenylate cyclase activity and phosphoinositide hydrolysis (119, 120, 121).

Specific ANF binding sites have been revealed in a variety of tissues that have been studied such as, the kidney, adrenals, smooth muscle cells, vascular endothelium, lungs and many other tissues, (195, 196). Initially, radioligand binding studies characterized ANF receptors as belonging to single class of receptors. Later on, however, affinity cross-linking and photoaffinity labeling studies demonstrated the existence of several distinct ANF binding sites in most tissues and cells (197, 198, 199).

Functional classification was made by studying the binding characteristics and the *in vivo* effects of a variety of truncated ANF analogs; these studies revealed that biologically active ANF bound to specific receptors and was associated with cGMP stimulation; the truncated analogs failed to elicit cGMP response although they also bound with high affinity (199, 200, 201, 202). Moreover, intravenous injection of these truncated analogs into anesthetized animals lead to increased plasma irANF, diuresis and natriuresis; these discrepancies between the *in vitro* and *in vivo* findings suggested the

possibility of existence of silent ANF receptors that were not associated with the expression of ANF function (200, 201).

Thus, a constellation of data suggested, early on, the presence of at least two subpopulation of ANF receptors; one class mediated the physiological function of ANF while another one was biologically inactive (119). The receptors also behaved differently on SDS-gel electrophoresis; biologically silent receptors migrated to about 130 and 65kDa under nonreducing and reducing conditions, respectively, indicating disulfide linked subunits (119, 198, 200, 203); the functional receptor migrated to 130kDa under both reducing and nonreducing conditions (204). The latter was then shown to copurify with particulate *guanylyl cyclase* over several chromatographic steps and interaction of ANF with this receptor activated particulate *guanylyl cyclase* (205).

Subsequent research lead to the purification and subsequent cloning and sequencing of three distinct receptors for the natriuretic peptides; two of these are linked to *guanylyl cyclase* (GC) designated GC-A and GC-B in order of their discovery; the third one which is not linked to *guanylyl cyclase* is designated ANF-C or clearance receptor. GC-A, GC-B and ANF-C receptors have also been designated $ANFR_1$, R_3 and R_2 receptors, respectively (206)

1.9.1 *Guanylyl cyclase*-A (GC-A) receptors:

Following the reports of ANF stimulation of particulate *guanylyl cyclase* and its copurification with the ANF receptor, Chinkers *et al.* (207) in 1989, reported the cloning of a novel membrane *guanylyl cyclase* (GC-A) from rat brain and human placental cDNA libraries using a cDNA probe based upon the sea urchin clone; these investigators found

that GC-A bears close homology with particulate *guanylyl cyclase* from the sea urchin; it is a single polypeptide chain with four domains: an extracellular domain, a transmembrane domain, a protein kinase like domain and a cyclase catalytic domain. Expression of the isolated cDNA into Cos-7 cell and cultured endothelial cells lead to increased basal as well as ANF-stimulated cGMP levels; ANF binding and cross-linking studies identified it as 130kDa protein (207, 208).

1.9.2 *Guanylyl cyclase*-B (GC-B) receptors:

The use of low stringency hybridization screening procedures of the rat brain and human placental cDNA libraries with the GC-A clone led to the isolation of the full length clone for GC-B receptor (209). GC-B receptors exhibit approximately 72% homology with GC-A in the protein kinase like and catalytic domains, whereas, there is only 43% identity within the extracellular domain, perhaps indicative of different ligand specificities (210). Indeed, when cells were transfected with either GC-A or GC-B and then stimulated with ANF or BNP the dose response pattern was different with the two receptors (211). Cos-7 cells transfected with GC-A responded to both ANF and BNP in a dose dependent fashion. However, ANF was more potent than BNP in stimulating cGMP; EC_{50} of ANF was 3 nM and that of BNP 25 nM. On the other hand, cells transfected with only GC-B receptors responded to ANF and BNP at the μM range; BNP elevated cGMP levels several fold higher than did ANF (121, 208, 211, 212). It appeared from these and other results that ANF was the specific ligand for GC-A receptors.

Although BNP appeared to more specific for GC-B, it was obvious that it was not its natural ligand because of the relatively high concentration of BNP needed to elicit a

response. This suggested the possible existence of a yet unidentified natural ligand for GC-B. Not very long after this, CNP was identified as a new member of the natriuretic family and several studies demonstrated that it possessed a very high affinity for GC-B and was the most potent stimulator of GC-B receptors (121, 212, 213, 214). Therefore, the currently accepted rank order of selectivity for natriuretic peptides to GC-linked receptors is as follows (fig. 1.7): ANF > BNP > > CNP for GC-A; CNP > BNP ≥ ANF for GC-B; ANF > CNP ≥ BNP for ANF-C (121, 214).

1.9.3 Other ANF receptors

Another 180-kDa membrane protein with *guanylyl cyclase* activity was also purified to apparent homogeneity from rat adrenocarcinoma cells; it bound ANF with 1:1 stoichometric ratio suggesting the coexpression of ANF binding site and *guanylyl cyclase* activity (215). This 180-kDa protein might represent a distinct ANF receptor; this was suggested by experiments in renal glomeruli which showed in affinity cross-linking two ANF binding bands, one at 130 and another at 180-kDa; similar studies in the inner medulla revealed only the 130-kDa. Western blot analysis using antiserum against the 180-kDa from rat adrenocarcinoma cells labeled a protein in the glomeruli and the antibody inhibited ANF stimulated cGMP production (216, 217), suggesting its presence in the glomeruli.

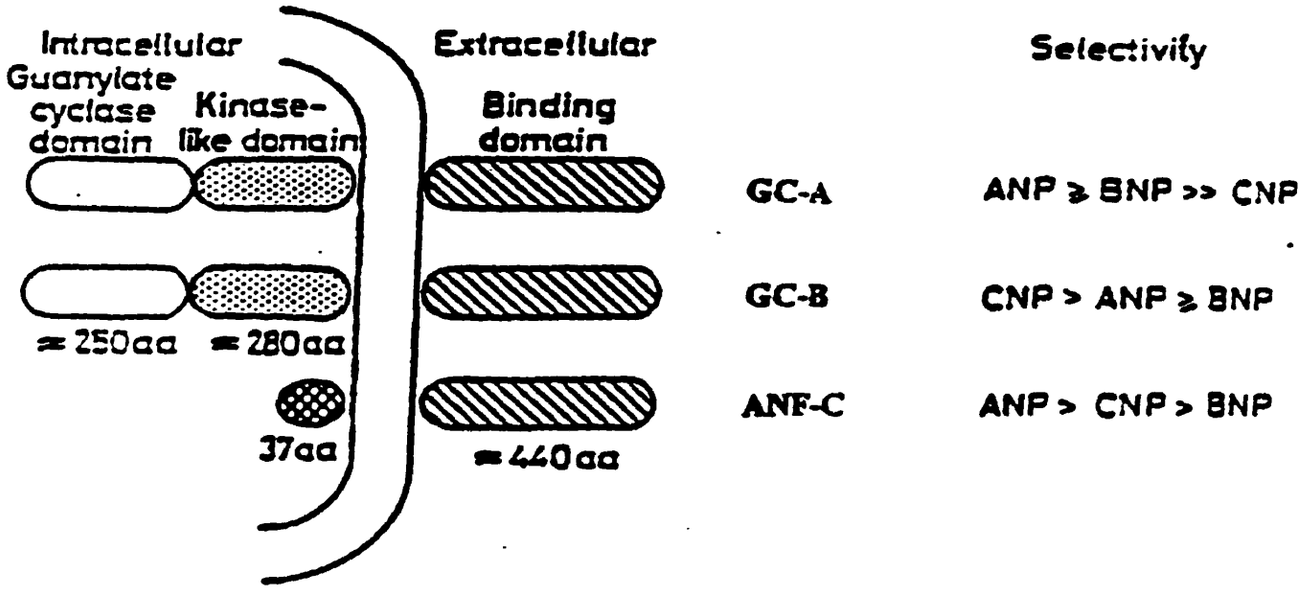


Fig. 1.7. Schematic diagram showing the natriuretic peptide receptors and their selectivity for the natriuretic peptides.
(Taken from ref. 121).

However, Identification of the gene encoding the protein will be required to establish with absolute certainty whether it represents a distinct ANF receptor or is the same as the currently known ANF receptors associated with other membrane proteins.

1.10 REGULATION OF GC-LINKED RECEPTORS:

Although the exact regulatory mechanisms of regulation of GC-linked receptors have as yet to be identified, several studies (218, 219) cast some light on this aspect. It appears that GC-A normally exist as an aggregate of homomer and that unlike other peptide receptors GC-A do not need ligand binding to form dimers or tetramers; however, receptor dimerization is essential for cyclase activity (205, 220, 221, 222). Mutagenesis studies revealed that the kinase like domain was not required for ANF binding as well as cyclase activity; infact mutants which expressed GC-A that lacked the kinase like domain showed increased basal cGMP generation than the wild type (223). Thus, the kinase like domain suppresses basal cyclase activity and acts as a negative regulator and the binding of ANF or ATP to receptors releases this inhibition (218, 224).

Another possible mechanism by which the GC-linked receptors are regulated is receptor desensitization by dephosphorelation (219, 225). When mouse fibroblast NIH3T3 cell were transfected with full length cDNA for GC-A and preincubated with ANF or PMA, the cGMP response to either 1 μM ANF or 1 μM ANF plus PMA was attenuated; the inactive analog of phorbol ester 4α-PMA was without effect; the effect was the same with crude membranes from cell extracts showing that the reduced response was not due to receptor internalization (225). Potter and Garbers (225), proposed two

models for this desensitization, homologous desensitization in the presence of ANF and heterologous desensitization through a protein kinase C-dependent mechanism. Pressor hormones such as arginine vasopressin, angiotensin II and endothelin which activate PKC through stimulation of phospholipase C have been shown to reduce ANF dependent cGMP generation; similarly, phorbol esters (PMA) which activate PKC attenuate the ANF-dependent cGMP production (226, 227, 228).

1.11 ANF-C OR CLEARANCE RECEPTORS:

Analogs of ANF with amino acid deletions in the carboxy terminal or within the ring were found to bind with high affinity to the majority of ANF binding sites in the kidney cortex, glomeruli, endothelial cells and vascular smooth muscle cells without inducing cGMP production; furthermore, these analogs did not elicit any renal or vascular effects in the isolated perfused rat kidney, but induced diuresis, natriuresis, and hypotension and raised plasma irANF when given to intact anesthetized animals (200, 201, 212, 229). Maack *et al* (200), thus, suggested that the majority of renal glomerulaar ANF receptors are biologically silent and act as a hormonal buffer system to modulate plasma ANF levels; they designated these receptors as clearance receptors.

The clearance receptor has been purified to homogeniety; its cDNA has been cloned and expressed in Xenopus oocyte (230,). ANF-C is a plasma membrane associated protein composed of an extracellular ligand biding domain that bears 35% homology with GC-A and GC-B receptors and a short cytoplasmic tail with no *guanylyl cyclase* activity (230). Nussenzveig *et al* (231) demonstrated that, ANF-C receptors clear ANF by a process of internalization followed by lysosomal hydrolysis of the peptide; these workers

suggested that receptor recycling occurs in a manner similar to other clearance receptors such as the low density lipoprotein receptors (LDL-R). and the process was constitutive.

However, the concept that ANF-C receptors function only as a clearance receptor is not universally accepted (232). Several workers (233, 234, 235), have reported that ANF inhibits adenylate cyclase and hence cAMP generation by acting on ANF-C receptors. Both ANF and ANF_{4-23} (a truncated ANF analog that binds only to the clearance receptors) suppressed adenylate cyclase activity in several tissues including platelets (which contain only NP-RC), rat aorta, brain, anterior pituitary and adrenal cortical membranes (235, 236). Anand-Srivastava *et al* (237), reported that the inhibition of adenylate cyclase by ANF is mediated by ANF-C through an interaction with a pertussis toxin-sensitive inhibitory guanine nucleotide regulatory protein (G_i).

1.12 TISSUE DISTRIBUTION OF ANF RECEPTORS:

ANF receptors are distributed widely in various tissues (238). Northern blotting and cDNA cloning have demonstrated the expression of GC-A receptors in the kidney, adrenal gland vascular and smooth muscle cells, brain and other tissues (239). Abundant expression for GC-B receptors was observed in the brain, adrenal medulla and pituitary (239). In addition, RT-PCR microlocalization of mRNA for GC-coupled ANF receptors in rat kidney demonstrated that GC-A receptors are expressed widely along the nephron (240); however, the highest intensity was detected in the IMCD, the major site of action of ANF. Although GC-B mRNA are expressed in the renal papillae, ligand binding and other studies did not detect expression of the protein for GC-B receptors (241 242). The distribution of GC-linked natriuretic peptide receptors seem to correspond to the central

and peripheral effects of these peptides in body fluid homeostasis; it also suggests different roles for the two receptors as well as divergent regulation.

CNP is the specific ligand for GC-B receptors; the distribution of its receptors in central tissues together with the fact CNP is abundant in central tissues explains the neuromodulatory role of CNP (212).

The peripheral distribution of GC-A receptors and particularly in the kidneys corresponds to the high affinity it exhibits for ANF and to the diuretic and natriuretic effects of ANF (214).

ANF-C receptors also show wide tissue distribution but the vascular and the renal glomeruli are rich in this receptor; this pattern distribution is consistent with a major role of these receptors as a ligand clearance receptors (119, 212).

1.13 ANF METABOLISM:

ANF has a very short half-life (119, 243, 244). The half-life ($t_{1/2}$) of intravenously injected ANF in animals and humans is about 2-4 min. After incubation of ANF in various tissue homogenates, Tang *et al* (245), found the following order of degradation: kidney > liver >lung > plasma > heart.

1.13.1Neutral endopeptidases (NEP; 24.11):

Current evidence suggests that ANF metabolism involves enzymatic degradation and receptor mediated internalization and hydrolysis.

The brush border of the renal proximal tubules is rich in neutral endopeptidase activity. NEP also known as enkephalinases are membrane associated zinc

metalloendopeptidases ectoenzymes that degrade many other peptides besides ANF such as insulin, kinins and enkephalins (246).

Incubation of ANF with membrane preparations from rat and rabbit kidney cortex led to a rapid loss of its vasorelaxant activity. HPLC analysis of the degradation product revealed that ANF was cleaved within the 17 amino acid ring at Cys^7 -Phe^8 and this was completely prevented by phosphoramidon (189, 247, 248). Sonnenberg *et al* (249), purified the protease from rat kidney cortex and showed that the purified enzyme cleaved ANF specifically at the Cys^7 -Phe^8 bond; this activity was inhibited by thiorphan and phosphoramidon.

In vivo studies, have also shown that infusion of phosphoramidon or thiorphan (both potent inhibitors of NEP activity) was associated with diuresis, natriuresis, hypotension and an increase in urinary cGMP excretion (250, 251), providing evidence that besides clearance by C-receptors ANF is also degraded by NEP.

1.13.2 Receptor mediated metabolism:

In addition to degradation by NEP, ANF is also removed from circulation by binding to ANF-C receptors as mentioned earlier.

1.14 BIOLOGICAL EFFECTS OF ANF

ANF produces diverse biological effects. Besides causing diuresis and natriuresis, ANF relaxes vascular and nonvascular smooth muscles, suppresses aldosterone secretion and inhibits cell proliferation (119).

1.14.1 Renal actions of ANF :

The kidney is a major site of action of ANF. The presence of ANF receptors coupled to *guanylyl cyclase* at multiple site along the nephron raises the possibility that ANF may exert its effects on various segments along the renal tubule (252). ANF increases GFR, alters renal hemodynamics, inhibits renin release and increases renal tubular water and sodium excretion (119).

It has been suggested that ANF increases GFR and filtration fraction by causing dilatation of preglomerular (afferent) arterioles and constriction of postglomerular (efferent) arterioles leading to increased pressure within the glomerular capillaries (253). Another additional determinant of GFR and filtration fraction is the glomerular capillary ultrafiltration coefficient K_f (a function of the surface area available for filtration and hydraulic permeability of glomerular capillaries); it increased markedly after the addition of ANF to isolated perfused glomeruli and to glomeruli precontracted with angiotensin II, demonstrating that ANF induces relaxation of the mesengial cells (254). Subsequent work (255) has shown that these cells possess specific high affinity ANF receptors and respond to ANF with marked elevations in cGMP.

The effects of ANF on the glomeruli leads to an increase in the filtered load of water and solute. Some investigators (256) proposed that the increase in GFR alone could account for the natriuresis and diuresis induced by ANF. However, several studies (257, 258) have revealed that ANF can produce diuresis and natriuresis without producing detectable alterations in GFR; in several of these studies, infusion of ANF at low doses resulted in diuresis and natriuresis without increasing GFR. Moreover, in the toadfish,

which lack glomeruli, ANF induced a striking natriuresis demonstrating that ANF can produce natriuresis by mechanisms independent of an increase in GFR (259). It should also be noted that increases in water and sodium delivery to the distal segments of the nephron following ANF infusion are by themselves insufficient to fully account for the observed diuresis and natriuresis (257, 260).

Clearance and micropuncture studies (261, 262), have shown that ANF caused increased lithium and phosphate excretion as well as increased solute delivery out of the proximal tubule; these observations were interpreted by some workers (262) that ANF inhibited sodium reabsorption along the proximal tubule; it was, however, argued (263) that these effects were due to increased filtered load rather than a direct action of ANF on the proximal tubule. In agreement with the latter view are the observations that ANF receptors were not detectable in microdissected proximal tubule segments and ANF did not stimulate cGMP production in proximal tubular cells (264, 265). However, ANF inhibited ANG II stimulated volume reabsorption in the proximal tubules; these data suggest that the ability of ANF to reduce fluid reabsorption in this segment of the nephron may be dependent on the basal transport rate and that dopamine plays a permissive role in the action of ANF in this segment of the nephron (261, 266). Currently, there is no clear evidence that ANF modulates salt and water transport in the loops of Henle and distal convoluted tubules; these segments are devoid of ANF receptors and ANF stimulated cGMP production is either absent or far more less than that observed in glomeruli and inner medullary collecting ducts (IMCD) (119, 265).

There is strong evidence that ANF produces diuresis and natriuresis by acting on the collecting duct cells. The renal collecting ducts seem to be the main target of several other hormones and they are generally recognized as the renal site where these hormones interact to achieve a fine control of renal water and salt excretion (267). Water reabsorbtion in this segment is principally under vasopressin control, which increases water permeability of the epithelium through its action on adenylate cyclase-linked V_2 receptors. Another hormone involved in the regulation of sodium reabsorbtion is aldosterone which increases sodium transport across the principal cells of the collecting duct by activation of an apical sodium channel and stimulation of basolateral Na^+-K^+ ATPase (Na^+ pump) activity (267).

1.14.2 Regulation of sodium reabsorbtion in the collecting ducts by ANF

A fixed fraction of the filtered load of fluid and solute is delivered to the collecting duct system (268). The constancy of solute delivered, results from two opposing processes. Tubuloglomerular feedback is the process that maintains GFR by sensing the concentration of sodium chloride reaching the distal tubule. Glomerulotubular balance is the process by which proximal fluid and sodium chloride reabsorbtion is readjusted so that a relatively constant fraction of the glomerular filtrate is reabsorbed. These two mechanisms act in concert so that generally 5-6% of filtered load reaches the collecting ducts (268). Normal sodium homeostasis requires the excretion of less than 1% of the filtered load (268). Therefore, the changes in sodium excretion normally take place as a consequence of the regulation of the transport of the 5-6% of the filtered load of sodium reaching the collecting ducts.

ANF regulates sodium reabsorption along the collecting ducts through both direct and indirect mechanisms (119); it acts directly on the collecting duct epithelium and inhibits sodium reabsorbtion. Studies on isolated perfused tubule have demonstrated that the outer medullary collecting ducts are not a major site of sodium transport (269) and almost all the sodium reabsorbtion takes place in the cortical and IMCD. These are the same segments that exhibit the highest ANF stimulated cGMP production and the presence of only *guanylyl cyclase*-linked ANF receptors (228, 265). ANF inhibited transport dependent oxygen consumption in the IMCD (270); the use of amiloride, ouabain and omphotericin in these preparations revealed that ANF inhibited Na^+ entry into these cells. Furthermore, application of ANF in whole cell patch-clamp preparations from primary cultures of rat IMCD reduced the open time of the amiloride-sensitive cation (Na^+) channel, providing direct confirmation that ANF acts by inhibiting Na^+ flux across an amiloride-sensitive channel in IMCD cells (271).

The mechanisms by which ANF inhibits conductive Na^+ entry also have been characterized (271, 272); ANF binds to GC-coupled receptors and stimulates cGMP production; ANF inhibits sodium reabsorption directly by binding to the cation channel and indirectly by activating cGMP dependent protein kinase (Fig. 1.8). In freshly prepared IMCD cells increasing cGMP by exposure to sodium nitroprusside (which stimulates soluble *guanylyl cyclase*), inhibition of phosphodiestrase or the addition of the membrane-permeable cGMP analog 8-bromo-cGMP mimics the inhibitory effects of ANF on transport-dependent oxygen consumption and on the uptake of sodium via conductive sodium channels (273). Also the application of cGMP to cell attached membrane patches from IMCD cells reduced the open time of the amiloride-sensitive

cation channel. Taken together these findings establish that cGMP is the mediator of the regulation of Na^+ transport in the IMCD by ANF (252, 272).

ANF acts on the zona glomerulosa cells of the adrenal gland to inhibit aldosterone secretion (274). Through its action on the macula densa cells of the juxtaglomerular apparatus, ANF inhibits renin release which leads to a suppression of the renin-angiotensin II-aldosterone-axis (275). Thus, ANF is indirectly involved in the fine regulation of collecting duct sodium transport through its influence on aldosterone secretion.

ANF is also known to antagonize the action of vasopressin in increasing water permeability in IMCD; this action contributes to its diuretic effect (276). In isolated perfused rat IMCD, application of ANF to the basolateral, but not to the apical surface inhibited vasopressin stimulated water reabsorption; ANF did not alter the cAMP response to vasopressin in this segment, although it inhibited the water permeability in response to cAMP analog, suggesting that ANF inhibits vasopressin mediated water permeability at a site beyond cAMP formation (276).

Another mechanism which contributes to the diuretic and natriuretic effects of ANF is changes in the transepithelial driving forces in the renal medulla. It has been suggested that ANF increases the hydraulic pressures in the loops of Henle, collecting ducts and vasa recta, causing a greater increase in pressure in the vasa recta than within

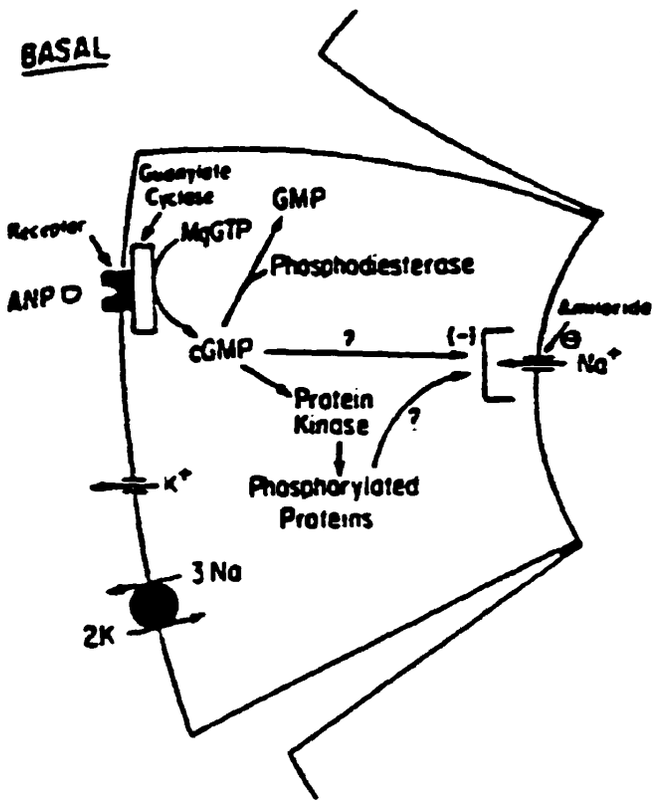


Fig. 1.8. Mechanism of Na^+ reabsorption and ANF action in IMCD cell. (Taken from ref. 119).

the adjacent papillary lumina (277); this effect alters the pressure gradient favoring net sodium excretion. In addition, ANF increases vasa recta blood flow leading to solute washout and dissipation of the gradient which decreases solute reabsorption in the medullary interistitium and thence to the capillaries (278). However, it is not clear weather medullary solute washout is a consequence or the cause of the natriuresis and diuresis induced by ANF.

1.14.3 Actions of ANF on the cardiovascular system:

ANF decreases blood pressure by reducing peripheral vascular resistance and cardiac output. Studies on the effects of ANF on vascular resistance have yielded conflicting reports. ANF relaxed precontracted aortic rings but it failed to relax precontracted mesenteric and cerebral arteries (279). Likewise, ANF did not alter coronary and mesenteric blood flow, although it reduced renal vascular resistance. However, ANF reduced peripheral vascular resistance in dogs under sympathetic blockage (280), implying that the response of resistance vessels to ANF depends on the autonomic tone in the animal.

ANF infusion leads to a decrease in stroke volume and cardiac output while heart rate usually decreases or fails to rise despite a marked fall in blood pressure; these effects are believed to result from a modulation of the autonomic nervous system by ANF. Rats infused with atrial extracts exhibited marked falls in blood pressure, heart rate, stroke volume, and cardiac output (281, 282). However, prior vagotomy and denervation of the carotid sinus in vagotomized animals was associated with less hypotensive effect, no decrease in heart rate and an augmentation of the stroke volume (283, 284); this indicates

that ANF can inhibit sympathetic activity by activating vagal afferents while at the same time indirectly stimulate sympathetic activity (vagotomy unmasks this effect) at the baroreceptor level by reducing blood pressure (119, 282). Additional factors involved in the reduction of cardiac output by ANF include reduction in preload i.e. a decrease in central venous return and hence right atrial pressure secondary to dilatation of the capacitance veins, increased resistance to venous return and increased transduction of plasma from the intravascular to the extravascular spaces (119, 284).

1.15 THE ROLE OF ANF IN PATHOPHYSIOLOGICAL CONDITIONS

ANF was the first endogenous substance to be identified as an agent that increases renal sodium excretion. Following its discovery a body of knowledge accumulated describing ANF as an important hormone that is intimately involved in fluid and electrolyte homeostasis and attempts were made to elucidate the role that ANF might play in various physiological and pathophysiological conditions (4, 119, 285). At the same time expectations were raised that pharmacological manipulations of the natriuretic peptide system might have therapeutic potentials in conditions associated with acute or chronic fluid and electrolyte disorders such as congestive heart failure (CHF), renal diseases and hypertension.

1.15.1 Hypertension:

With the development of specific radioimmunoassay for ANF it became possible to study the release of the peptide in humans as well as animals. In the spontaneously hypertensive rats (SHR), plasma ANF levels are higher than in WKY control rats (286),

even before overt hypertension develops; intracerebroventricular infusion of ANF selectively attenuated the exaggerated salt appetite of SHR compared to that of WKY rats (287). However, the binding of ANF to its receptors in vascular smooth cells was similar between SHR and WKY rats although the cGMP response to ANF was blunted in SHR (288). Similarly, in experimental models of hypertension, such as the one-kidney, one-clip and DOCA-treated rats both plasma ANF and ANF gene transcription are increased (136, 289, 290). In humans with hypertension plasma ANF levels vary widely (291). The benefits of ANF in the treatment of hypertension were variable depending on the dose; lower doses of ANF have little effect on blood pressure whereas higher doses produced intolerable hypotension (292, 293, 294); these studies also demonstrated that ANF has a low therapeutic index which might limit the therapeutic potential of ANF.

1.15.2 Heart failure:

Patients with congestive heart failure exhibit high levels of plasma ANF; the levels of ANF closely correlate with the severity of heart failure, varying directly with right atrial pressure and pulmonary wedge pressure (295). Animal models of congestive heart failure also behave similarly. In addition, ventricular ANF gene expression is noted in animal models and humans with CHF (143, 295).

Despite the elevated circulating ANF levels, patients with CHF show evidence of volume overload, increased preload and increased peripheral vascular resistance (295, 296) suggesting an acquired insensitivity to endogenous ANF. Nevertheless, studies (296, 297) have demonstrated that ANF modulates the renal, hemodynamic and endocrine effects of CHF; also infusion of anti-ANF antibodies to rats with CHF due to myocardial

infarction attenuated renal salt and water excretion, implying that volume overload would have been worse were it not for the modest diuresis and natriuresis from the high levels of circulating ANF (298).

1.15.3 Renal diseases:

End-stage renal disease leads to sodium retention and edema formation (285). In humans with volume overload secondary to renal failure, pre-dialysis plasma ANF levels were twice the post-dialysis levels. Normovolumic individuals with renal disease did not exhibit significant elevations of the peptide (299) thus implicating the natriuretic peptide in the maintenance of fluid homeostasis in progressive renal failure. Infusion of ANF into rats with reduced renal mass led to diuresis and natriuresis; this effect was abolished with specific anti-ANF antibodies (300) suggesting that there might still be a functional reserve to further augment GFR and sodium excretion. However, in nephrotic syndrome, which also is characterized by sodium retention, plasma ANF levels are low (301) and rats with experimental nephrosis exhibited a blunted diuretic and natriuretic response to infused ANF (302), thus raising questions about the role of ANF in this pathophysiological state.

There is a wealth of information regarding the role of ANF as a hormone involved in volume homeostasis; whether it is truly a physiological substance, however, has not been resolved (303). This uncertainty arises from the fact that plasma levels of ANF do not correlate with diuresis and natriuresis under all circumstances. For example, left atrial distention in conscious dogs resulted in natriuresis and two-fold increase in plasma ANF; similar atrial distention following cardiac denervation produced comparable ANF levels

but did not result in natriuresis (304), thus dissociating ANF secretion and sodium excretion.

Currently, there is no known clinical syndrome of ANF excess or deficiency. Moreover, experimentally induced ANF deficiency in the autoimmune rat model (303) and ANF excess in transgenic mice (194) did not lead to perturbations in sodium balance in these rats. Similarly, the renal response to ANF infusion in cirrhotic dogs exhibiting ascites and avid sodium retention was variable; ANF produced natriuresis in some but not all cirrhotic dogs (305). The above observations raise important questions about the role that ANF might play in states of chronic volume expansion and sodium retention. Some workers (303) have suggested that ANF might be important in states of acute volume expansion but not in chronic states.

1.16 ADRENOMEDULLIN:

Adrenomedullin is a newly discovered hormone that was identified on the basis of its ability to elevate cAMP levels in a platelet bioassay (306). The peptide consists of 52 and 50 amino acid residue in humans and rats, respectively, with intermolecular disulfide bond and shows close structural homology with Calcitonin gene-related peptide (CGRP) (306, 307). Adrenomedullin is widely distributed in several peripheral tissues including adrenal medulla, kidney, lungs, endothelium, anterior pituitary and central nervous system (308, 309); the cDNA encoding the precursor for human and rat adrenomedullin has been cloned and sequenced (307, 310).

Adrenomedullin has several important biological effects that seem to be related to fluid, electrolyte and cardiovascular homeostasis; it has a potent hypotensive effect which

seems to be due to the generation of nitric oxide by the vascular endothelium in response to the peptide (311). Indeed, it has been demonstrated that adrenomedullin decreases vascular resistance in the mesenteric, pulmonary and hind limbs in rats and cats; nitric oxide synthase inhibitors such as L-NAME decrease this effect (311, 312). Adrenomedullin also is a potent natriuretic agent which acts both by increasing renal blood flow and direct tubular action. Intra-arterial infusion of adrenomedullin in dogs resulted in an increase in renal cortical and medullary blood flow as well as in a marked diuretic and natriuretic responses; these effects were attenuated by nitric oxide synthase inhibitors (313, 314), suggesting that the renal action of adrenomedullin might be mediated by nitric oxide. Adrenomedullin also suppressed aldosterone secretion in response to ANG II and potassium (315). Thus, available evidence indicates that adrenomedullin resembles ANF in its biological effects and like the latter might have a role in fluid and electrolyte homeostasis. However, to our knowledge the effects of adrenomedullin in pregnancy have not been reported.

1.17 ANF AND PREGNANCY

As mentioned earlier, pregnancy is associated with marked volume expansion and cumulative sodium retention. Under normal conditions even a milder state of fluid expansion would lead to an increase in circulating plasma ANF and suppression of the renin-angiotensin-aldosterone-axis (1, 28). However, plasma ANF exhibit little change during pregnancy, both in humans and animal models (316, 317, 318). Steegers *et al* (317), investigated control and pregnant women, both in the sitting and left lateral position and found no difference in plasma ANF between the two groups; these authors

also found that ANF degradation was not increased during pregnancy. Similarly, Kristensen et. al. (319) also, did not observe any difference in ANF activity from the hearts of virgin and pregnant rats.

Despite the fact that pregnancy-induced volume expansion does not lead to changes in circulating ANF levels, maneuvers which stimulate ANF release are still operative during pregnancy. Nadel *et al* (318) found that acute increase in sodium load by saline infusion stimulated ANF release in pregnant rats and suppressed the renin-angiotensin II aldosterone-axis. On the other hand, chronic volume expansion by deoxycorticosterone acetate (DOCA) in uninephrectomized pregnant rats was not sensed by the atria and did not lead to ANF release; this DOCA-induced volume expansion was detected by renal volume sensors and led to suppression of the renin-angiotensin-aldosterone system (318). Similarly, circulating ANF levels decreased in pregnant women on a low sodium diet (317).

Given the dramatic increase in the ECFV that accompanies pregnancy, it can be said that this volume expansion is not sensed as such by the atria probably because the expansion is accommodated by an enlarged maternal compartment; consistent with this is the fact that atrial ANF secretory response to appropriate stimuli is still intact (318). In the immediate postpartum period, however, following the abrupt decrease in the size of the maternal vascular compartment that results from the expulsion of the placenta and the autotransfusion of blood from the emptied uterus, plasma ANF levels is markedly elevated (318, 320). The increase in ANF in the immediate postpartum period is accompanied by a decrease in plasma renin activity, aldosterone and the increased

diuresis and natriuresis of the early puerperium; this is associated with a restoration of the expanded vascular volume to prepregnant levels (318, 320, 321).

It should be mentioned that in pregnancy-induced hypertension (pre-eclampsia) which is characterized by a contraction of the intravascular volume and a lesser increase in cardiac output, plasma ANF levels are higher than in normal pregnancy (320). After delivery, ANF levels in women with pre-eclampsia decreases significantly as the blood pressure normalizes (320, 322). Fievet et. al.(323), have found significant positive correlation between plasma ANF and mean arterial pressure and reported from sequential data that plasma ANF increases as the patient becomes hypertensive.

The pathophysiology of pre-eclampsia as well as the mechanisms that lead to high ANF levels in this state of relative vasoconstriction is poorly understood. There is no over stimulation of the renin-angiotensin system in pregnancy-induced hypertension as the renin-angiotensin-aldosterone system is comparable to that in normal pregnancy (316); however, there is loss of the vascular hyporesponsiveness to ANG II, which is characteristic of normal pregnancy (324). It is postulated that the high ANF levels in pre-eclampsia might prevent further rise in blood pressure by virtue of the hypotensive action of ANF as well as by promoting diuresis, shift plasma volume into the interstitial space and decrease renin and aldosterone secretion (316).

1.18 Modulation of the effects of ANF by pregnancy

As described earlier, the major effects of ANF are diuresis, natriuresis, suppression of aldosterone and renin secretion and a decrease in blood pressure. Neither

of these effects of ANF are conducive to the plasma volume expansion and the high aldosterone levels seen in pregnancy. This suggests that some mechanisms operate during gestation to counter the physiological effects of ANF. Studies in our laboratory have revealed a significant refractoriness to the aldosterone-suppressant (324a) and to the tocolytic effects (324b) of ANF by pregnancy. Moreover, our laboratory demonstrated that both pregnancy and progesterone downregulated adrenal and myometrial ANF receptors.

Based on these and other observations the following hypothesis was formulated:

An attenuation of the renal effects of ANF might be required to allow physiologically needed plasma volume expansion during pregnancy.

The study presented in this thesis was designated to test this hypothesis using pregnant rats as the experimental model.

CHAPTER TWO: MATERIALS AND METHODS

2.1 CHEMICALS:

Anti-ANF GC-A and GC-B receptor antibodies were a gift from Dr. D. L. Garbers, Howard Hughes Medical Institute and Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX.

The following chemicals were purchased: IPTG, L.B. broth base, LB agar, ultra pure agarose, X-gal, dATP, dCTP, dGTP, dTTP, phenol-chloroform-isoamyl alcohol 25:24:1| [v/v], S.O.C. medium (GIBCO Burlington, ON); acrylamide, PIPES (1,4-piperazinediethanesulfonic acid), tris-base (Boehringer Mannheim, Montreal, Que.); ampicillin, aprotinin, BSA (bovine serum albumin), creatine phosphate, creatine phosphokinase, GTP, HEPES (N-[2-hydroxyethyl]-piperazine-N'-[2-ethanesulfonic acid]), IBMX (1,-3-isobutylmethylxanthine), leupeptin, MOPS (3-[N-morpholino]propanesulfonic acid), N-lauroylsarcosine (sarkosyl), estradiol-17β, pepstatin, PMSF (phenylmethylsulfonylfluoride), progesterone, tris-HCl, DEPC (diethyl pyrocarbonate), glycerol,

2-mercaptoethanol, nonidet P-40 ((octylphenoxy)-polyethoxyethanol), triton X-100 (t-Octylphenoxypolyethoxyethanol), Tween-20 (polyoxyethylene sorbitan monolaurate) thiorphan, leucine aminopeptidase, phosphoramidon, and N-succinyl-Ala-Ala-Phe-7-amido-4-methyl-coumarin (SAMC) (Sigma, St.Louis, MO); ammonium persulfate, bromophenol blue, bis(N,N'-methylene-bis-acrylamide), glycine, xylene cyanole FF; TEMED (N,N,N',N'-tetra-methylethylenediamine) (Bio Rad, Hercules, CA);

rat ANF (rANF) $rANF_{1-28}$, $rANF_{4-23}$, and anti-ANF antiserum (Peninsula, Belmont, CA); ^{125}I-labeled ANF_{1-28} (2000 Ci/mmol), guanosine 3',5'-cyclic monophosphate (cGMP) radioimmunoassay (RIA) kits (Amersham, Oakville, ON); boric acid, EDTA (ethylenediaminetetraacetic acid), EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid), ethidium bromide, guanidine thiocyanate, urea, chloroform, formaldehyde, formamide, isopropyl alcohol, reagent alcohol and all other high purity chemicals (Fisher, Montreal, Que.).

2.2 BUFFERS

2.2.1 Electrophoretic buffers:

*5X TBE

54.0 g Tris base
27.5 g boric acid
20.0 ml 0.5 M EDTA (pH 8.0)
added 1L H_2O
Sterilized by autoclaving

*50X TAE

242 g Tris base
57.1 ml glacial acetic acid
100.0 ml 0.5 M EDTA (pH 8.0)
added 1L H_2O
Sterilized by autoclaving

MOPS
41.2 g MOPS
26.6 ml 3 M Sodium acetate pH 7.0
20.0 ml 0.5 M EDTA (pH 8.0)
Added 800 ml of DEPC-treated
H_2O, stirred and autoclaved. Then added MOPS and EDTA
and adjusted to pH 7.0 with ~1-2 g NaOH pellets
and made to 1 L H_2O (DEPC-treated and autoclaved)

*RNase-free buffers prepared by using autoclaved DEPC-treated H_2O

2.2.2 Common buffers

0.5 M EDTA (pH 8.0)
186.1 g EDTA
Adjusted pH with ~20 g NaOH pellets
added 1 L H_2O
Sterilized by autoclaving

TE (pH 8.0)
10 mM Tris-HCl (pH 8.0)
1 mM EDTA (pH 8.0)
Sterilized by autoclaving

*20X SSC

175.3 g NaCl
88.2 g Sodium citrate
Adjusted pH to 7.0 with 10 N NaOH
Added 1 L H_2O
Sterilized by autoclaving

*20X SSPE

175.3 g NaCl
27.6 g NaH_2PO_4-H_2O
7.4 g EDTA
Adjusted pH to 7.4 with 10 N NaOH
added 1L H_2O
Sterilized by autoclaving

Phosphate buffered saline (PBS)

5.38g NaH_2PO_4/L H_2O
16.35g Na_2HPO_4/L H_2O
9.0g NaCl/L H_2O

*RNase-free buffers prepared by using autoclaved DEPC-treated H_2O

2.2.3 Gel loading buffers

6X DNA gel loading buffer

30% Glycerol
0.25% bromophenol blue
0.25% xylene cyanol FF
in TAE or TBE

Sequencing gel loading buffer
98% deionized formamide
10 mM EDTA (pH8.0)
0.025% bromophenol blue
0.025% xylene cyanol FF

RNA formaldehyde gel loading buffer

50% Glycerol
1mM EDTA
0.25% bromophenol blue
0.25% xylene cyanol FF

RNase protection assay loading buffer
95% deionized formamide
1X TBE
0.025% bromophenol blue
0.025% xylene cyanol blue

2.2.4 Other buffers

ANF receptor assay buffer

50 mM Tris HCl (pH 7.40
5 m M $MgCl_2$
0.2 mM PMSF
0.1% bacitracin
0.2% BSA

cGMP determination buffer

10 mM HEPES (pH 7.4)
5 mM KCl
1 mm Na_2HPO_4
1.2 mM $MgSO_4$
5.5 mM glucose
6 mM L-alanine
1 mM IBMX

2.3 ANIMALS:

Sprague - Dawley rats (Charles Rivers, St. Constant, Quebec) were used for these studies according to a protocol of the McGill University Animal Care Committee. Animals were housed at 22 - 24^0 C, 55 - 70% humidity, and 12h light (07 00 - 19 00) - 12h dark schedule, and fed *ad libitum* rat chow and tap water. Female rats weighing 175 - 200 g were housed with 250 - 275 g male rats over night and the presence of sperm in the vaginal wash the following morning indicated day 0 of pregnancy. A separate group of 225 - 250 g female rats were used as virgin control to match the weight of 13 - 15 day pregnant rats. A group of 225 - 250 g virgin rats were injected subcutaneously for three consecutive days with 2 mg/kg/day progesterone. Some of the pregnant rats were allowed to deliver and were used on postpartum day 2-3.

A separate group of rats were ovariectomized (OVX) under ether anesthesia; 10-14 days following the removal of ovaries rats were injected subcutaneously once a day for three days with peanut oil (vehicle), 0.1 mg/kg estradiol-17β or 2 mg/kg progesterone or a combination of estradiol-17β and progesterone; these group of animals together with virgin control and pregnant rats were used to study the renal effects of bolus injection of ANF.

2.4 ANESTHESIA

In preliminary studies, rats were anesthetized with 45 mg/kg pentobarbital sodium; however, the prolonged duration of these studies required supplementation of the anesthetic in the middle of data collection, and supplementary injections of pentobarbital sodium altered the blood pressure. All experiments reported in these studies

with the exception of pharmacokinetic studies were therefore done under urethane (1 g/kg) anesthesia; with this approach no anesthetic supplementation was needed. Body temperature of the animal was maintained at 37^0 C by an overhead lamp.

2.5 CANNULATION

The jugular vein was cannulated with PE50 catheter for bolus injection or infusion. The carotid and femoral arteries were cannulated with PE50 catheters for monitoring blood pressure (by means of Statham pressure transducer P32) and for collecting blood samples, respectively. The urinary bladder was cannulated with PE90 catheter for the collection of urine.

2.6 DETERMINATION OF PLASMA VOLUME AND INULIN SPACE:

Plasma volume was determined by the Evan's blue dye-dilution technique (322). Briefly, 0.3 ml of 3 mg/ml dye was injected intravenously; a 15 minute period was allowed for equilibration, following which 1 ml of blood was collected. The concentration of the dye in plasma was determined by measuring absorbance at 620 nm.

To determine inulin space (ECFV), 2 μCi [^{3}H]inulin was injected intravenously, and blood samples were collected at 2, 5, 10, 15, 30, and 60 min. Volume of distribution (V_d) of inulin was estimated from the regression line of log plasma inulin concentration vs time. The V_d of inulin was the total dose injected divided by the plasma inulin concentration at time 0 (estimated by extrapolating the concentration/time curve to time 0).

2.7 DETERMINATION OF THE RENAL AND HEMODYNAMIC EFFECTS OF ANF:

Following the cannulations, an infusion of 0.9% saline at a rate of 5.5 ml/Kg/h via the jugular vein cannula was started by a Harvard infusion pump. After 30 - 40 min of stabilization period, 0.9% saline containing 2 μCi/ml [^{3}H]inulin was infused for 90 min Two 20-min urine samples were collected into preweighed Eppendorf tubes; at the mid point of each urine collection, 0.2 ml blood was withdrawn from the femoral artery for measuring glomerular filtration rate (GFR). This was followed by an infusion of ANF at a rate of 1nmol/kg/h for a 30-min period, the infusion rate was increased to 2 nmol/kg/h for the next 30 min and then to 4 nmol/kg/h for another 30 min. Urine was collected during the last 20-min of each infusion period, and blood samples for the measurement of GFR and plasma ANF were collected at midpoint of each urine collection period.

Separate experiments were done to determine the modifications of the renal response to ANF by truncated ANF_{4-23}, a specific ligand for the clearance receptors (200), and by thiorphan, an endopeptidase inhibitor (249). In the first set of studies, ANF_{4-23} was first injected as a bolus dose of 25 nmol/kg, followed by an infusion of 36 nmol/kg/h for 30-min; at the end of the 30-min infusion was composed of 36 nmol/kg/h/ ANF_{4-23} plus 1 nmol/kg/h ANF. In the second set of experiments, thiorphan was infused at 24 μmol/kg/h for 30-min, followed by an infusion of thiorphan plus 1 nmol/kg/h ANF. Urine and blood samples were collected as described above. At the end of experiment rats were killed by injection of excess air.

2.7.1 Calculation of GFR

GFR was calculated as the clearance of inulin: [(urinary inulin concentration × urine volume/min)/plasma inulin concentration].

2.7.2 Urinary sodium and potassium

Urine sodium and potassium were measured by an IL - 443 Flame Photometer.

2.7.3 Urinary cGMP

urinary cGMP was measured by RIA, using a commercial kit (Amersham); urine samples were first acetylated and cGMP standard curve was constructed in the range of 2-128 fmol/tube. cGMP in urine was detected using high specific activity [^{125}I]2'-O-succinyl-cGMP tyrosine methyl ester together with a highly specific and sensitive antibody. Separation of bound from free antibody was achieved with a second antibody, Amerlex-M.

2.8 ANF ASSAYS:

Blood samples were collected into heparinized tubes containing aprotinin, and plasma was separated by centrifugation and stored at -70 °C until assayed.. For ANF assays samples were thawed and extracted on C_{18} Sep-Pak cartridges; the efficiency of ANF recovery was determined by ^{125}I-ANF and was > 80%. Immunoreactive ANF (irANF) was determined by RIA using ^{125}I-labeled ANF and an anti-rANF antisera that did not react with ANF_{4-23} (225). Briefly, the elutes from the Sep-Pak columns were dried, reconstituted in phosphate saline buffer pH 7.4 and then incubated overnight at 4

°C with the tracer and antibody; goat anti-rabbit gamma globulin and normal rabbit serum were added and the incubation continued for another 2 h followed by the addition of polyethyline glycol and centrifugation to separate the bound from the free ligand.

2.9 ANF PHARMACOKINETICS:

Virgin and 20 -day pregnant rats were anesthetized by pentobarbital sodium (40 mg/kg ip). The left jugular vein and carotid artery were cannulated for injections and blood collections, respectively, as described before. ^{125}I-ANF (1μCi) was injected as bolus dose and blood (0.2ml) was collected into heparinized syringes at 0.25, 0.5, 1, 2, 3, 5, 7, and 10 min; plasma was separated mixed with aprotinin and stored at -80^0 C until assayed.

Initially, we attempted to separate intact ANF by precipitation with trichloroacetic acid (TCA) as utilized by others (229, 326). However, in our hands, TCA precipitate contained some fragmented ANF. Therefore, we used separation of intact ^{125}I-ANF by high pressure liquid chromatography (HPLC) and quantified it as previously described (327). Briefly, plasma samples were passed through C_{18} Sep-Pak cartridges, and the material retained in the cartridges was eluted with 3 ml aqueous acetonitrile (80%) containing 0.1% trifloroacetic acid (TFA). After the removal of the acetonitrile, samples were mixed with authentic unlabeled rANF and loaded on to a C_{18} μBondapak column (Waters, Milford MA). Samples were chromatographed by HPLC using a linear gradient of 10 - 60% aqueous acetonitrile (80%) containing 0.1% TFA throughout for 60 min at a flow rate of 1 ml/min. ANF was identified by means of an ultraviolet detector at 215 nm, and the fraction corresponding to ANF was collected and radioactivity counted.

Plasma half life, clearance, and volume of distribution were calculated from plasma concentration versus. time curve by conventional techniques (325).

2.10 RENAL METABOLISM OF ANF:

Plasma membranes from the renal cortex were prepared according to a previously described method (248). Briefly, rats were decapitated, and the kidneys quickly removed and placed in ice-cold phosphate buffered saline (pH 7.4). The cortical portion was dissected, minced with scissors, and placed in a homogenization buffer (pH 7.4) containing 10 mM Na_2HPO_4, 1 mM $MgCl_2$, 30 mM NaCl, 1 mM dithiothreitol, 5 μM PMSF, 0.02% NaN_3, and 10 ng/ml DNase. The tissue minces were homogenized by means of a Polytron homogenizer (Brinkmann, Rexdale, ON) three times each time for 15 s at a setting of 7.

The homogenate was then centrifuged at 1000 g for 5 min, and the supernatant was layered on top of a 41% sucrose solution (vol/vol in the homogenization buffer) and centrifuged at 95,000 g for 1 h. The pellet was washed twice with the buffer to remove the sucrose and centrifuged at 95,000 g for 30 min. Aliquots of membrane preparations (1 μg protein in 50 μL) were preincubated at 37^0 C in a shaking water bath for 10 min, following which ANF was added to achieve a concentration of 50 μM. Incubation was stopped by adding 400 μL of 0.1% TFA at different time periods. Samples were then centrifuged immediately at 13,000 g for 5 min; the supernatant was aspirated, dried in a speed-Vac, and stored at -80^0 C until assayed for intact ANF by HPLC as described previously (327). ANF was quantified on the basis of peak area using an on-line integrator (Waters).

In order to verify that ANF was metabolized by neutral endopeptidase (NEP), 25 μM phosphoramidon, a potent NEP inhibitor (328), was added at the beginning in two experiments from each group of rats; cortical membranes were incubated with 50 μM ANF in the presence or absence of phosphoramidon and the reaction was terminated in 1 h and intact ANF assayed by HPLC as described above.

2.11 RENAL NEUTRAL ENDOPEPTIDASE (NEP) ACTIVITY:

Measurement of NEP activity in renal cortical membrane was done on the basis of cleavage of the fluorogenic succinyl-amido-methyl-coumarin (SAMC) to phe-amidomethyl-coumarin (PAMC) as described by other workers (329). Briefly, membrane preparations were first diluted 1:50 in 6% albumin (W/V); this was then diluted 1:10 in physiological saline, which was again diluted 1:300 in 50 mM tris(hydroxymethyl)aminomethane-HCl buffer (pH 7.6) containing 1% Triton X. Membrane samples (1.2 μg protein/ml) were incubated with 8.3 μM of the substrate SAMC at 37^0 C for 1 h. For the blanks, the mixture was incubated with 25 μM phosphoramidon. The reaction was terminated by adding phosphoramidon and heating the samples at 95^0 C for 15 min. The reaction mixture was then incubated with 5 μg/ml leucine aminopeptidase at 56^0 C for 1 h.

The intensity of the amidomethyl-coumarin fluorescence was measured by means of a Perkin-Elmer LS 30 fluorometer at wavelengths of 367 nm and 440 nm, respectively. NEP activity was estimated from the difference between the fluorescence of samples and corresponding blanks. To quantify NEP activity, a standard curve was constructed by incubating serial dilutions of PAMC with 5 μg/ml leucine aminopeptidase at 56^0 C for 1

h and recording the fluorescence as above. NEP activity was therefore expressed as the rate of formation of PAMC (nmol/min/μg protein).

2.12 ASSAY OF ANF RECEPTORS BY RADIOLIGAND BINDING:

For each experiment, kidneys from 3-4 rats were placed in ice-cold phosphate buffered saline (PBS pH 7.4) and were bisected longitudinally; cortex and papillae were dissected and pooled separately. Glomeruli were prepared by the graded sieving technique (330). Briefly, the cortex was finely minced with scissors; the suspension was then passed through stacked sieves and washed with ice-cold PBS. Glomeruli were harvested from the top of a 75 μm sieve and centrifuged at 2,000 g for 10 min at 4^0 C. Purity was assessed under light microscopy and was > 90%.

Minced glomeruli and papillae were homogenized with a Polytron homogenizer at a setting of 7 and two pulses each of 30 s duration in a buffer (pH 7.4) containing 0.25 mM sucrose, 50 mM tris-HCl, 5 mM $MgCl_2$, and 1 mM EDTA. The homogenate was centrifuged at 5,000 g for 10 min at 4 ^{0}C , and the supernatant recentrifuged at 100,000 g for 1 h (331). The pellet was resuspended in the assay buffer (pH 7.4) containing 50 mM Tris-HCl, 5 mM $MgCl_2$, 0.2 mM PMSF, and 0.1% bacitracin and stored at -80^0 C until used within one week.

ANF binding assays were performed as described previously (331, 332). Briefly, membranes were thawed and diluted with an assay buffer containing 0.2% BSA. Aliquots of 25 μg protein were incubated for 2 h at room temperature in a MultiScreen Assay System (Millipore, Mississauga, ON) with 10 pM [^{125}I]-ANF in the absence or presence of increasing concentrations of unlabeled ANF_{1-28} (binds to both ANF-GC and ANF-C

receptors) or ANF_{4-23} (binds to only ANF-C receptors) in a final incubation volume of 0.1 ml. Separation of bound from free was achieved by rapid vacuum filtration and washing five times with 0.15 M NaCl.

Plates containing the membranes were dried and punched into 12 x 75-mm polystyrene tubes and the radioactivity was counted in an LBK Clinigamma counter (Wallac) with a counting efficiency of >80%. The binding in the presence of 10^{-6} M unlabeled ANF was taken as nonspecific binding. The maximal binding capacity (B_{max}) and the apparent dissociation constant (K_d) were calculated by nonlinear analysis of displacement curves using a Lundon ReceptorFit competition Ligand program (Chagrin Falls, Ohio); displacement of $^{125}IANF_{1-28}$ by unlabeled ANF_{1-28} and ANF_{4-23} was subjected to homologous and heterologous analysis, respectively.

2.13 cGMP PRODUCTION BY GLOMERULI, MEDULLA AND PAPILLAE:

Effects of ANF on cGMP production by glomeruli and papillae were determined as previously described (265, 333). Glomeruli and papillae from 3-4 rats were prepared as described before and used to construct a concentration-response curve to ANF; five such experiments were done with each group of animals. In addition, four separate experiments were done to measure the effect of a single concentration (100 nM) of ANF on cGMP production in virgin and postpartum rats.

Glomeruli were prepared as described above. Papillae were finely minced with scissors and suspended in a buffer (pH 7.4) containing in (mM): 135 NaCl, 5KCl, 1 Na_2HPO_4, 1.2 $MgSO_4$, 5.5 glucose, 6 L-alanine, and 10 HEPES equilibrated with 95%

O_2-5% CO_2. Tissues were then subjected to collagenase (1mg/ml) digestion for 90 min at 37^0 C in a shaking water bath following which papillary suspensions were filtered through four layers of gauze and centrifuged at 1000 g for 10 min. The pellet was then washed four times with the above buffer to remove the collagenase, and viability was assessed with Trypan blue exclusion.

Glomeruli and papillary cell suspensions (20 μg protein/100 μl) were preincubated in the assay buffer containing 1 mM 3-isobutyl-methylxanthine (IBMX) at 37 ^{0}C for 10 min in a shaking bath. Tissues preparation were then incubated with increasing concentrations of ANF for 10 min; the reaction was stopped by the addition of 0.5 ml acidic ethanol. Samples were centrifuged at 13,000 g for 5 min and the supernatant dried in Speed-Vac; cGMP in the supernatant was assayed using commercial radioimmunoassay kits (Amersham) as described before.

2.14 cDNA PROBES FOR RAT ANF RECEPTORS:

Sense and antisense primers were prepared according to published cDNA sequences of rat ANF GC-A, GC-B, and C receptors (208; 209). The primers were made complementary to the region of the sequences spanning exon 3 and 6 of the receptor subtypes. This region shows maximal heterogeneity and corresponds to the extracellular domain. The primer pairs (McGill biotechnological Sheldon Center) used for amplification were: 5'-CACTACCCCAAGCTACTGCGGGCCGTG-3' (HYPKLLRAV) as a sense and 5'-GCTGTGTCAGAACACAAATTATAC-3' (AVSEHKLY) as an antisense, encoding for a.a. 223 to 231 and 431 to 438, respectively, for ANF GC-A receptor; 5'-GTGTATACCCGAGAGCCAGG-3' (VYTREP) as a sense primer and 5'-

CTGAGAAGCAGATTTGGTGG-3' (EKQIWW) as an antisense primer corresponding to a.a. 202-207 and 415-420, respectively, for ANF GC-B receptor; 5'-TGCCTACAATTTCGACGAGAC CA-3' (AYNFDET) as a sense primer and 5'-GCTCTTTGAAATTGAGAATAGATGA-3' (SLKLRID) as an antisense primer, corresponding to a.a. 228-234 and 449-455, respectively, for ANF-C receptor. cDNA (1 to 2 μg) from rat lung lambda gt-10 library 5'STRETCH (Clontech Laboratories, Palo Alto, CA) was amplified using Taq DNA polymerase (2U) (GIBCO BRL, Burlington, ON) in 100 μl reaction buffer (20 mM tris-HCl, pH 8.4, 50 mM KCl , 2.0 mM $MgCl_2$, 0.25 mM each of dCTP, dGTP, dATP, dTTP and 0.5 μM each of the primers) for 35 cycles (each cycle was 94°C-30 sec; 60°C-30 sec; 72°C-1 min) for GC-A receptor; and 35 cycles (94°C-30 sec; 58°C-30 sec; 72°C-1min) for GC-B and C receptors.

The PCR fragments were inserted into a PCR II vector (Invitrogen, San Diego, CA) at a 1:3 molar ratio of vector:PCR product. Briefly, 25 ng of the PCR products were ligated using T4 DNA ligase (2.5 Weiss U) in 10 μl reaction buffer (50 ng PCR II vector, 6 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 5 mM NaCl, 0.1 mg/ml BSA, 7 mM 2-mercaptoethanol, 0.1 mM ATP, 2 mM DTT and 1 mM spermidine) at 14-15°C overnight. *Epicurian coli* Jm109 competent cells (Stratagene, La Jolla, CA), were transformed with 1-2 ng of the plasmids by heat shock (42°C, 90 s; 1 min on ice); SOC medium was added to the transformation vials which were further incubated at 37°C for 60 min. Subsequently, LB agar plates containing ampicillin (100 μg/μl) were spread with 25 μl of X-gal (40 mg/ml), and 20 μl of IPTG (40 mg/ml) for color selection (blue/white screening) and plated with 50 μl of the transformation vials; plates were put for overnight

incubation at 37°C; the efficiency of the transformation was approximately $1X10^6$ transformants/μg.

Selected white colonies were transferred to 3 ml of culture medium (L.B. broth containing ampicillin (100 μg/μl) and incubated in a shaking-bath (200 rpm) at 37°C for 5 h following which 10 ml of fresh medium was inoculated with 200 μl of the original cultures for overnight at 37°C. An aliquot of each culture was saved in a freezing storage medium (15% glycerol) and stored at -80°C. DNA was extracted from the cultures by alkaline extraction, using the QIA prep spin plasmid kit (Qiagen, Chatsworth CA). Bacterial cells were lysed in NaOH/SDS buffer; the lysates were neutralized and adjusted to high salt binding conditions with a KCl/guanidine-HCl containing buffer; following centrifugation, the supernatants were applied to QIAprep-spin columns. The columns were washed with wash buffers (70% ethanol, low salts) and DNA was eluted with TE pH 8.5. The clones were sequenced using a T7 sequencing kit (Pharmacia, Uppsala, Sweden) to determine the sequences of rat ANF GC-A, GC-B and C receptor partial cDNAs. Briefly, 1-2μg of template DNA was sequenced with the Sanger method of dideoxy chain termination (201), using SP6 or T7 as primers and [α-^{35}S]dATPαS.

2.15 ISOLATION OF TOTAL RNA:

For each set of experiments, tissues from 3-4 rats of the different groups were snap-frozen in liquid nitrogen and stored at -80°C. Tissues were homogenized with a Polytron in Trizol reagent (GIBCO, Burlington, ON). Chloroform was added to the samples and was followed by a centrifugation which sequestered RNA to the aqueous phase. Total RNA was precipitated with isopropyl alcohol and purified by phenol-

chloroform extraction. The RNA was re-precipitated with 100% ethanol (10% [v/v] 3 M sodium acetate pH 5.2), washed with 75% ethanol and redissoved in an RNase-free water. Quantification of total RNA was done by absorbance at 260 nm. To assess the integrity of the RNA and to make certain that equal amounts of samples were used, equivalent quantities of total RNA for each sample were electrophoresed on formaldehyde-agarose gels (0.5 μg/ml ethidium bromide); gels were visually inspected under UV light as a method of standardization.

2.16 SYNTHESIS OF ^{32}-P-LABELED ANTISENSE RNA PROBES:

Initially, [^{32}P]-labeled antisense RNA probes were synthesized using an *in vitro* riboprobe transcription kit (Promega, Madison, WI), which generated labeled RNA fragments of 647, 654 and 687 nucleotides for GC-A, GC-B and ANF-C receptors, respectively. These probes were intended for use in Northern blot hybridization; however, failure to optimize this technique in detecting the natriuretic peptide receptors prompted us to use the ribonuclease (RNase) protection assay which proved to be more sensitive. Therefore, in order to obtain RNase protection assay compatible RNA probes, the plasmids were digested with restriction endonucleases that produced "shorter" DNA templates; digestion of the plasmids with HindIII for GC-A, BstB1 for GC-B and DdeI for the C receptor, generated templates yielded labeled probes of 253, 272 and 352 nucleotides, respectively.

For transcription, approximately 1 μg of linearized template DNA was transcribed into RNA by 15-20 U of T7 RNA polymerase for ANF GC-A or SP6 RNA polymerase for GC-B and ANF C receptors in a final volume of 20 μl of a buffer 40 mM Tris-HCl

(pH 7.9) containing 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 20 U RNasin, 0.5 mM each of ATP, GTP and UTP, 12 μM CTP, and 50 μCi [α-^{32}P]CTP (3000 Ci/mmol). Samples were incubated for 60 min at 37°C; DNA templates were removed by digestion with DNase I (1U) for 15 min at 37°C following which the final volume was adjusted to 70 μl (0.3M sodium acetate, 0.15 μg/ml tRNA). This was followed by a phenol/chloroform extraction. Unincorporated nucleotides were partially removed from the labeled probe by ethanol precipitation (2.5 volumes of 100% ethanol); following centrifugation, the pellets were washed with 70% ethanol and the RNA was resuspended in 500 μl of hybridization buffer (see next section). Aliquots of 10 μl were used to measure the specific activities of the probes.

2.17 RNase PROTECTION ASSAYS

RNase protection assays were performed as described previously (334). Briefly, 20 μg of total RNA was incubated with $5X10^4$ cpm each of GC-A, GC-B and ANF-C receptor probes in 40 μl of hybridization buffer (80% deionized formamide, 40 mM PIPES, pH 6.7, 1 mM EDTA and 0.4 M NaCl) at 50°C for 12-16 h; following this, non-hybridized RNA was digested in 400 μl of digestion buffer (10 mM Tris-HCl, pH7.5, 1mM EDTA and 0.3 M NaCl) that contained ribonuclease A (10 μg/ml) (Boehringer Mannheim, Montreal, Que.) and ribonuclease T-1 (200 U/ml) (Boehringer Mannheim, Montreal, Que.) for 30 min at room temperature. The samples were further digested with proteinase K (2.5 mg/ml) (Boehringer Mannheim, Montreal, Que.) in sarkosyl solution (7.5% [w/v]) at 37°C for 30 min; protected fragments were purified with guanidine thiocyanate buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0 and 0.5%

sarkosyl) and precipitated with isopropyl alcohol. The protected fragments were separated on urea-6% polyacrylamide gels at 70 V over a 2 h period. The gels were dried under vacuum and the bands were visualized and quantified by phosphorimaging (Fujix Bio-imaging analyzer BAS-1000). In addition, gels were autoradiographed using Kodak-XO-matic film, and the exposures were scanned and quantified (Molecular Analyst); this enabled a confirmation of data from phosphorimaging.

2.14 WESTERN BLOTTING OF ANF GC-A AND GC-B RECEPTORS:

Western blots were essentially conducted as previously described by Potter and Garbers (227) using polyclonal anti-ANF GC-A and anti-ANF GC-B receptor antibodies. Tissues were homogenized in ice-cold buffer (40 mM KCl, 10 mM Hepes, pH 7.9, 3 mM $MgCl_2$, 5% glycerol [v/v], 0.5 μ/ml leupeptin, 0.1 μg/ml aprotinin, 1.5 μg/ml pepstatin and 100 μg/ml PMSF) with a polytron homogenizer for 15-20 s. The homogenates were centrifuged at 500 g for 10 min at 4°C and the supernatants were recentrifuged at 112,000g for 60 min at 4°C. The pellets were resuspended in 0.5 ml homogenizing buffer containing 0.5% [v/v] nonidet P-40. Aliquots of the solubilized membranes (40-60 *u*g) were dried in speed-Vac and resuspended in 20 μl sodium dodecyl sulfate (SDS) loading buffer (63 mM Tris-HCl, pH 6.8, 2% [w/v] SDS, 10% [v/v] glycerol, 0.0025% bromophenol blue and 2.5% β-mercaptoethanol).

In order to optimize the quality of the Western blots, we used an alternate procedure that involved purifying the protein preparation with a cellulose ion-exchange column. Tissues were homogenized in ice-cold buffer A (20 mM Tris-HCl pH 7.5, 0.5

mM EDTA, 0.5 mM EGTA, 25 μg/ml leupeptin, 25 μg/ml aprotinin, 1 mM PMSF) and centrifuged at 100 000 g for 60 min at 4°C. The pellets were rehomogenized on ice in buffer A containing 1% [v/v] Triton-X; the homogenates were again centrifuged at 100,000 g for 60 min and the supernatants were saved as the membrane fractions.

A column loaded with pre-swollen DE52 ion-exchange cellulose (Whatman, Fairfield, NJ) was washed with buffer B (20 mM tris-HCl pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA). Membrane preparations were applied to the columns by gravity; the columns were washed with buffer B and the proteins were eluted with buffer B containing 0.2 M NaCl. Since the protein concentrating step resulted in impaired migration of the bands during electrophoresis (probably due to high salt concentration), it was avoided in subsequent experiments. Protein samples were heated at 70°C for 15 min and loaded onto SDS polyacrylamide gels (Biorad, Hercules, CA) and separated by SDS-PAGE at 70 V over a 90 min period.

The protein bands were electrophoretically transferred to nitrocellulose membranes (Biorad, Hercules, CA) and the non-specific binding sites were blocked with buffer C (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.1% [v/v] Tween-20) containing 10% skimmed milk for 12 h at 4°C. Membranes were then incubated for 60 min at room temperature in buffer C containing 5% skimmed milk with anti-GC-A or anti-GC-B receptor rabbit antibodies (1:500 dilution); following this membranes were washed three times (10 min each) with buffer C and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibodies (1:1000 dilution) in buffer A containing 5%

skimmed milk for 60 min. The membranes were then washed three times with buffer C as described.

In addition, the quality of the Western blots was further improved by re-using the primary anti ANF GC-A or anti ANF GC-B solution a second time; this contributed in decreasing the background noise. The immunoreactive bands were visualized using an enhanced chemiluminescence kit (Amersham) and exposed on X-ray film for variable time periods.

2.19 PROTEIN DETERMINATION

All protein determinations were made by the dye binding method using bovine serum albumin (BSA) as standard (335).

2.20 STATISTICS

Difference between two means were compared by Student's t-test and multiple means were compared by one way analysis of variance followed by Bonferroni test for significance. A probability of less than 0.05 ($P < 0.05$) was assumed to denote significant difference. Throughout this thesis data are presented as means ± S.E.M.

CHAPTER THREE: RESULTS

3.1 PLASMA PROFILE OF ANF AND ALDOSTERONE.

In order to determine the plasma profile of ANF and aldosterone, blood was collected serially from the same animals starting before the onset of pregnancy and continuing during pregnancy and the postpartum period.

Plasma irANF was 163 ± 38 fmol/ml before the onset of pregnancy and significantly higher on day 6 (506 ± 56 fmol/ml); however, plasma irANF on days 13, 18, and 20 of gestation were not different from levels before the onset of pregnancy; there was a significant rise in plasma irANF on day 1 postpartum (527 ± 107 fmol/ml) and the levels remained elevated up to postpartum day 7 (fig. 3.1a).

Plasma aldosterone (fig. 3.1b) was 9.0 ± 1.3 pmol/ml before pregnancy and progressively increased from day 6 to reach a peak level of 37.1 ± 3 pmol/ml on day 20 of gestation; levels fell precipitously on postpartum day 1 and remained low on day 3 but increased again on postpartum day 7 (18 ± 1.7 pmol/ml).

3.2 RENAL EFFECTS OF ANF:

3.2.1 Basal values.

The mean blood pressure of both 13-15 and 21 day pregnant rats was lower than that of virgin rats. Consistent with the well-known physiological changes, pregnancy was associated with an increase in inulin space and plasma volume.

The GFR of 13-15 day pregnant rats was higher than the GFR of virgin control; GFR of progesterone-treated rats was comparable to that of pregnant rats and higher than the GFR of virgin rats.

Basal urine output was lower in 21 day pregnant rats than in any other group of animals (virgin, 13-15 pregnant, postpartum and progesterone-treated).

Basal values of inulin space, GFR and plasma volume of postpartum rats were generally comparable to values in virgin rats (Table 3.1).

3.2.2 Effects of ANF infusion on plasma immunoreactive ANF (irANF):

ANF (irANF) prior to the infusion of ANF did not differ between different groups of animals (Table 3.1; Fig. 3.2). Infusion of ANF caused a significant increase in irANF in all groups of rats. There was no significant difference in plasma irANF levels among different groups of animals at all infusion rates of ANF (1, 2 and 4 nmol/kg/h).

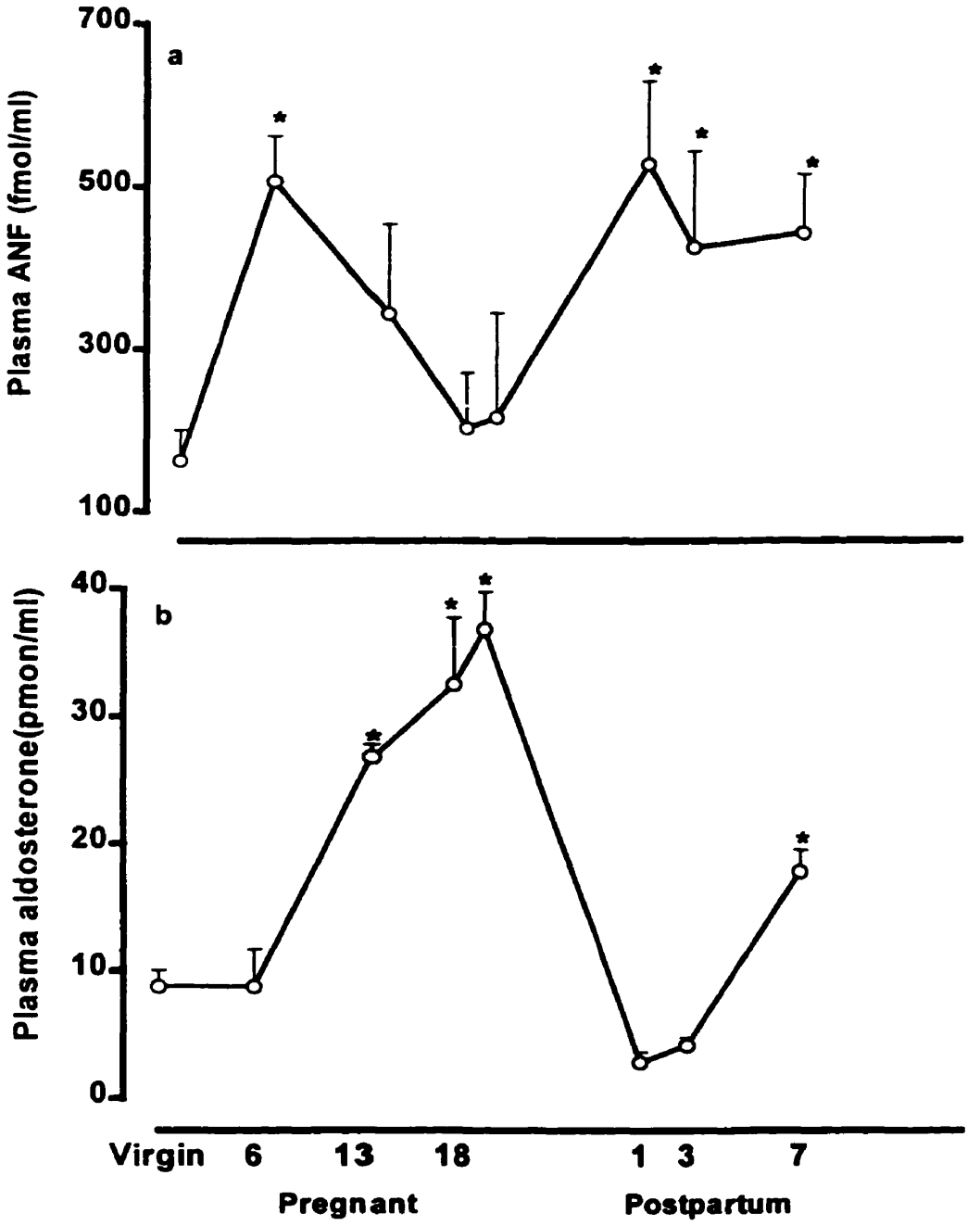


Fig. 3.1. Serial plasma ANF and aldosterone levels in rats before, during and after pregnancy; blood was collected under ether anesthesia.

Values are means ± SE of six rats. * Different ($P < 0.05$) from virgin values.

TABLE 3.1. Basal values in virgin, progesterone-treated, pregnant and postpartum rats

Variables	Virgin	Progesterone treated	Pregnant day 13-15	Pregnant Day 21	Postpartum day 3
n	17	6	18	6	6
Body weight (g)	239±4	235±2	258±5*	305±15*	261±11
Kidney weight (g)	2.17±0.09	2.32±0.20	2.21±0.06	2.20±0.06	2.33±0.12
MBP (mmHg)	117±3	113±4	102±2*	103±7*	108±7
V_{inulin} (ml)	56±2.1	not done	64±1.1*	69±1.3*	61±1.2
V_{plasma} (ml)	10.9±0.5	not done	14.5±0.7*	16.4±0.7*	12.7±0.4
P_{ANF} (fmol/ml)	72±7	75±10	80±7	76±8	84±13
GFR (ml/kg/min)	3.5±0.17	4.4±0.22*	5.2±0.27*	4.52±0.61	3.8±0.4
V(µl/kg/min)	30±2.7	28±4.5	20±1.5*	15±1.5*	19±1.6*
$U_{Na}V$(µmol/kg/min)	1.35±0.2	1.1±0.3	1.0±0.1	0.9±0.2	1.8±0.3
$U_{K}V$(µmol/kg/min)	3.1±0.5	2.4±0.7	1.6±0.6	2.4±0.7	5.3±0.4*
U_{cGMP} (pmol/min)	1.47±0.54	1.10±0.29	1.16±0.22	1.62±0.59	1.18±0.22

Values are means ± SE. MBP, mean blood pressure; V_{inulin}, volume of inulin; V_{plasma}, plasma volume; P_{ANF}, plasma ANF; GFR, glomerular filtration rate; V, urinary volume; $U_{Na}V$, urinary sodium; $U_{K}V$, urinary potassium; U_{cGMP}, urinary guanosine 3', 5'-cyclic monophosphate.

* Different ($p < 0.05$) from the values for virgin rats in the same row.

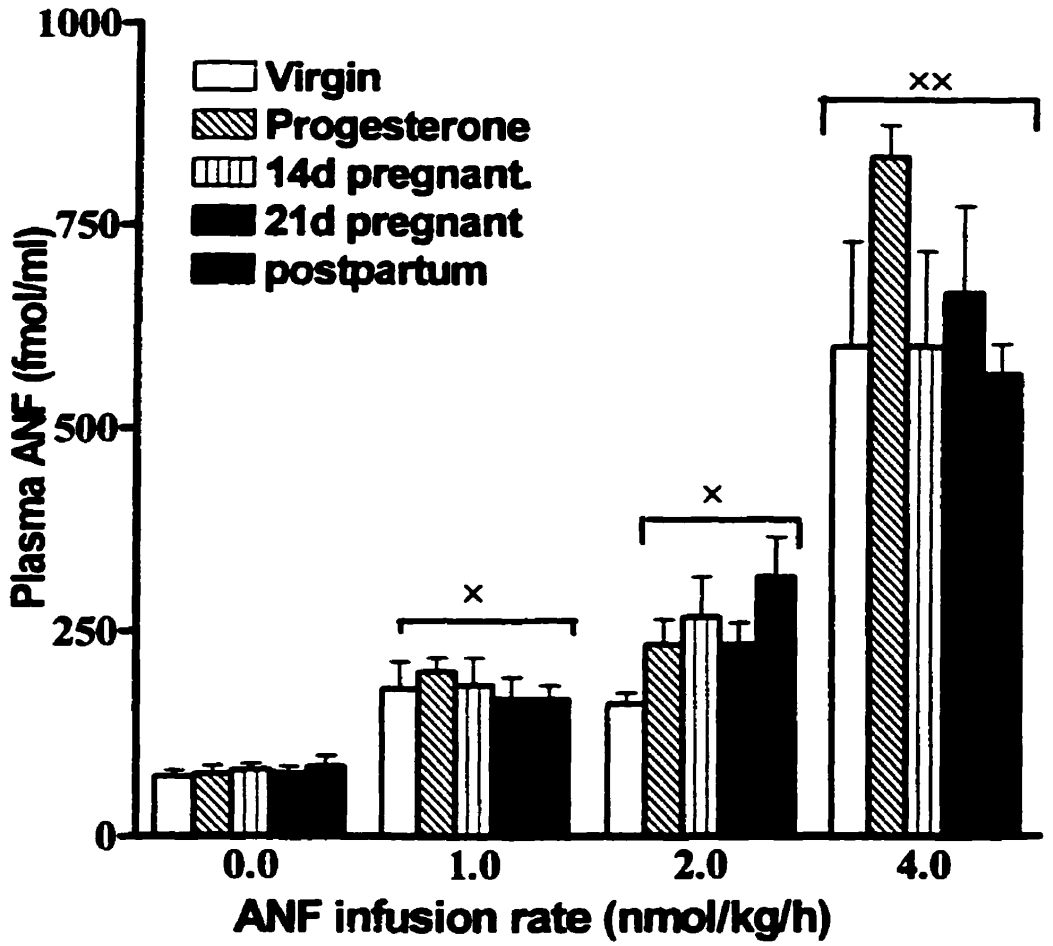


Fig. 3.2. Plasma irANF before and after ANF infusion in virgin, progesterone treated, *days 13-15* and *day 21* pregnant and *day 3* postpartum rats under urethane anesthesia.

Data are means ± SE of 6-10 experiments. x $P < 0.05$ compared with control values at 0 ANF; xx $P < 0.05$ compared with corresponding values at 0-2 nmol/kg/h ANF

3.2.3 Effects of ANF infusion on mean blood pressure:

The basal blood pressure of virgin rats was significantly higher than that of 13-15 day pregnant animals. ANF caused a decrease in blood pressure to a lesser extent in pregnant than in virgin rats such that mean blood pressure (MBP) following ANF infusion did not differ between different groups of animals (Fig. 3.3).

3.2.4 Effect of ANF infusion on GFR:

Basal GFR of 13-15 day pregnant and progesterone-treated rats was significantly higher than that of virgin rats (Table 3.1). Absolute GFR values in virgin, progesterone-treated, pregnant and postpartum rats did not differ following infusion of ANF (Fig. 3.4a). However, percent increases in GFR over basal following infusion of ANF in virgin and postpartum rats were significantly greater than in progesterone-treated and pregnant rats (fig. 3.4b). There was no difference in the effect of ANF on GFR of virgin and postpartum rats (fig. 3.4b).

3.2.5 Effects of ANF infusion on urine volume:

ANF caused a dose-dependent increases in urine output in virgin, progesterone-treated, pregnant as well as postpartum rats (Fig. 3.5). However, the diuretic effects of ANF were significantly ($P < 0.05$) less in days 13-15 as well as 21 day pregnant rats than in virgin rats (Fig. 3.5). There was no significant difference in the diuretic effects of ANF in postpartum and virgin rats and between progesterone-treated and virgin rats (Fig. 3.5).

3.2.6 Effects of ANF infusion on urinary sodium and potassium:

ANF caused a dose-dependent increase in urinary sodium and potassium excretion in all groups of animals (fig. 3.6). However, the diuretic and kaliuretic effects of ANF were significantly less in pregnant than in virgin rats. There was no difference in the natriuretic and kaliuretic effects of ANF in postpartum and virgin rats (fig. 3.6)

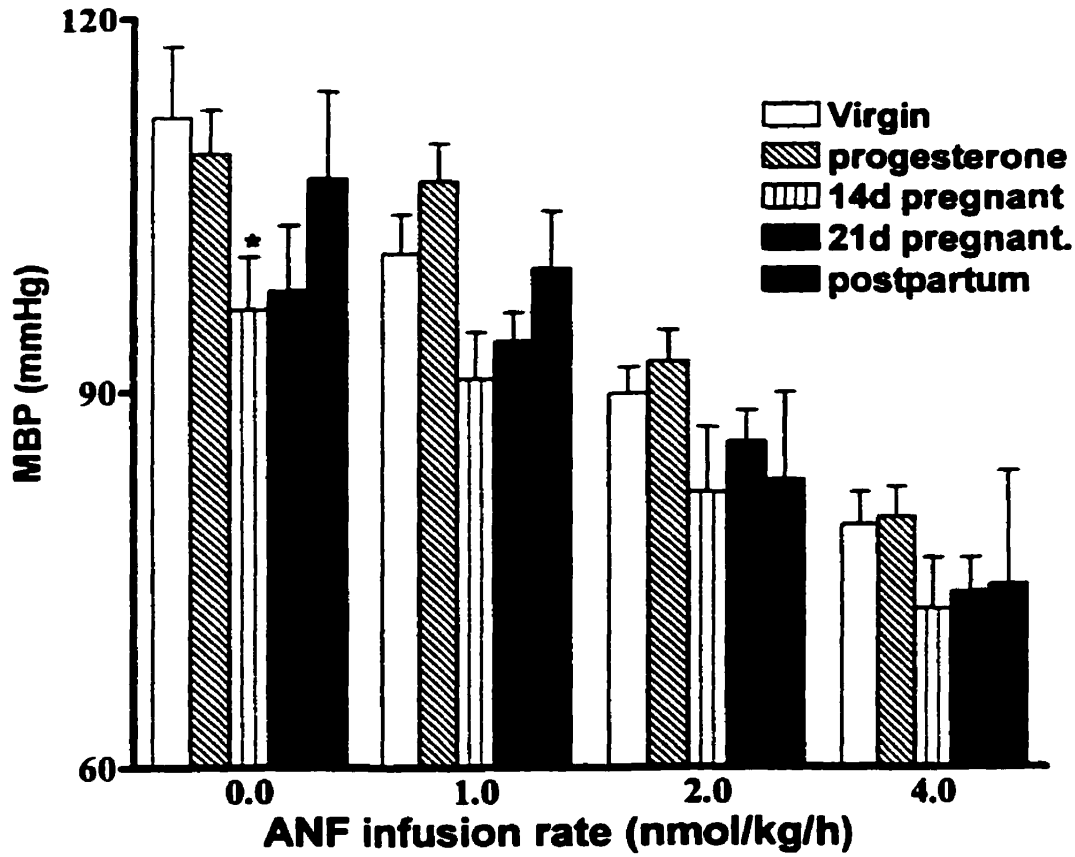


Fig. 3.3. Influence of constant infusion of increasing concentration of atrial natriuretic factor on mean blood pressure (MBP).

Virgin, progesterone treated (progesterone), days 13-15 (14d) and 21 day (21d) pregnant and 3 day postpartum rats under urethane anesthesia. Data are means ± SE of 6-10 experiments. * $P < 0.05$, different from values for virgin rats.

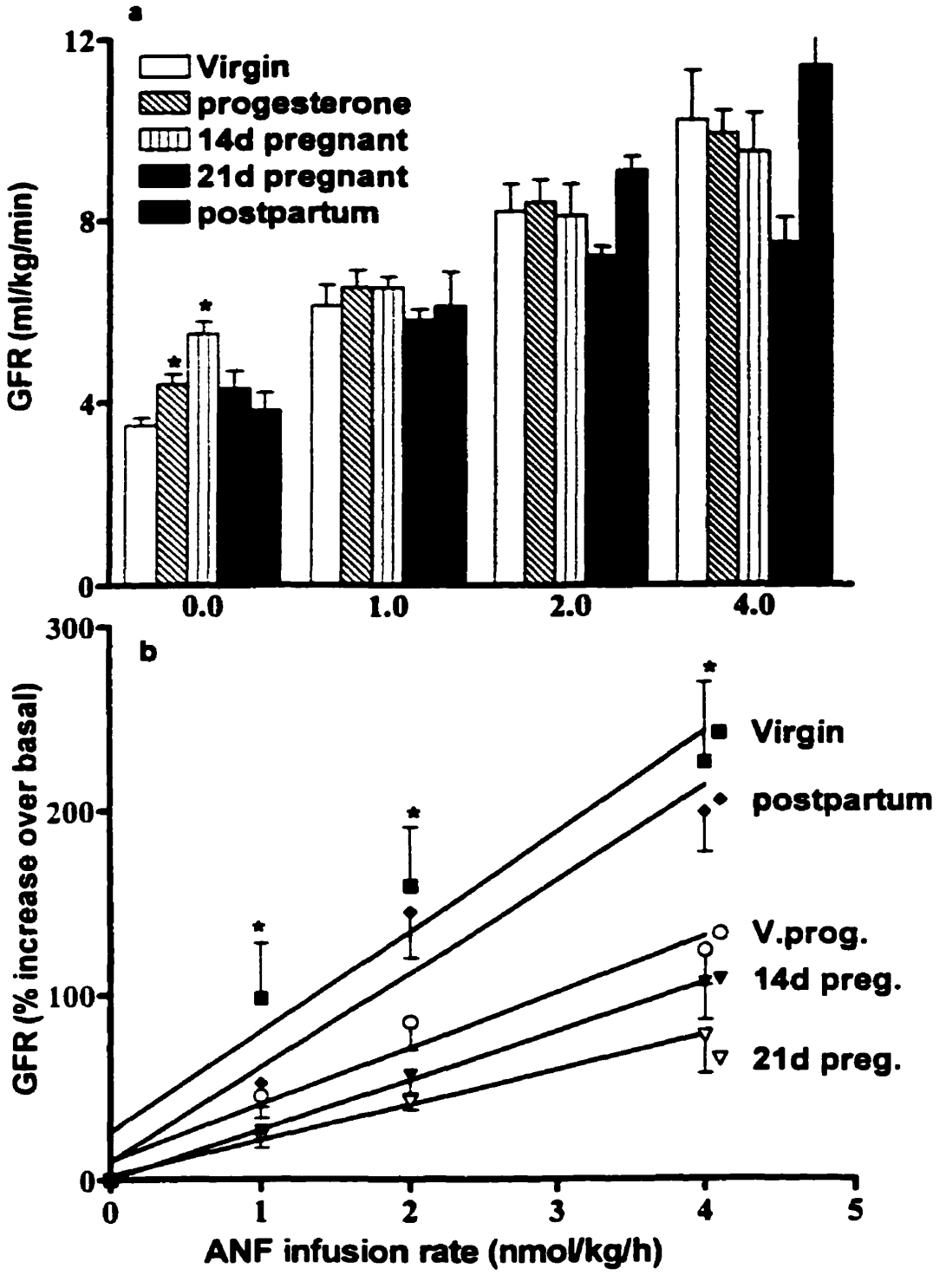

a
12
8
4
0
GFR (ml/kg/min)
Virgin
progesterone
14d pregnant
21d pregnant
postpartum
0.0
1.0
2.0
4.0
b
300
200
100
0
GFR (% increase over basal)
Virgin
postpartum
V.prog.
14d preg.
21d preg.
0
1
2
3
4
5
ANF infusion rate (nmol/kg/h)

Fig. 3.4. Influence of constant infusion of increasing concentration of atrial natriuretic factor on. absolute (a) and percent (b) increase over basal glomerular filtration rate (GFR).

Of virgin, progesterone-treated (progesterone), days 13-15 (14d) and 21 day (21d) pregnant and 3 day postpartum rats under urethane anesthesia. Data are means ± SE of 6-10 experiments. * $P < 0.05$, different from values for virgin rats.

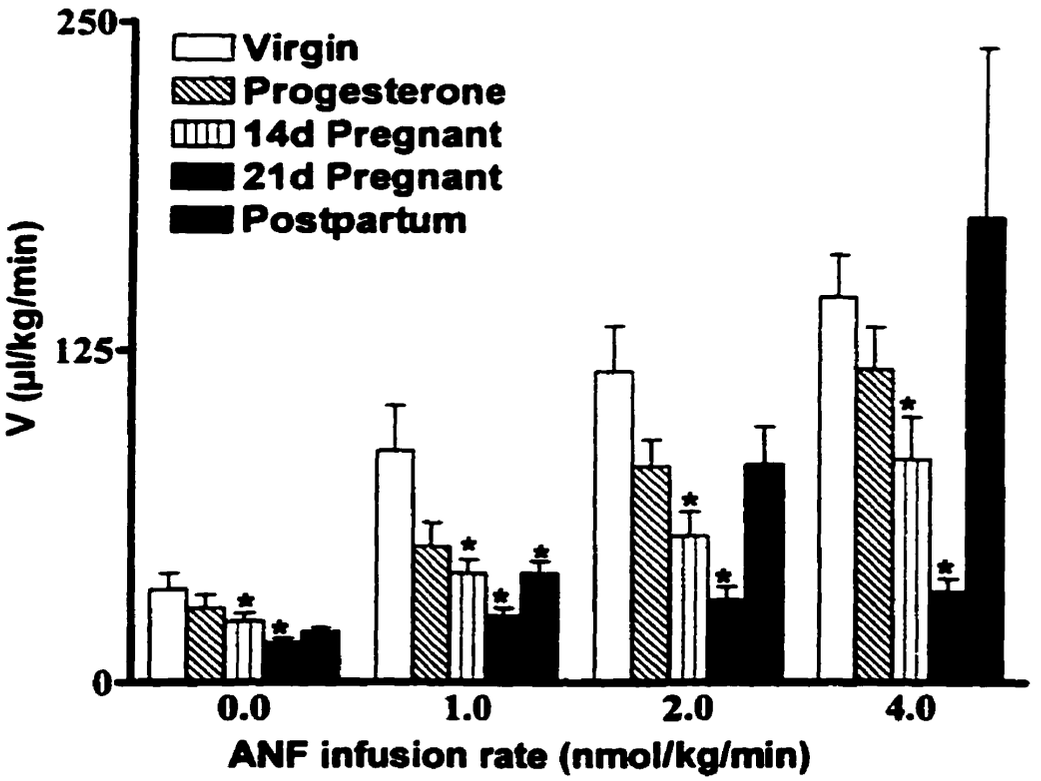


Fig. 3.5. Influence of constant infusion of increasing concentration of atrial natriuretic factor on urinary volume (V).

Of virgin, progesterone treated (progesterone), days 13-15 (14d) and 21 day (21d) pregnant and 3 day postpartum rats under urethane anesthesia. Data are means ± SE of 6-10 experiments. * $P < 0.05$, different from values for virgin rats at indicated dose of ANF.

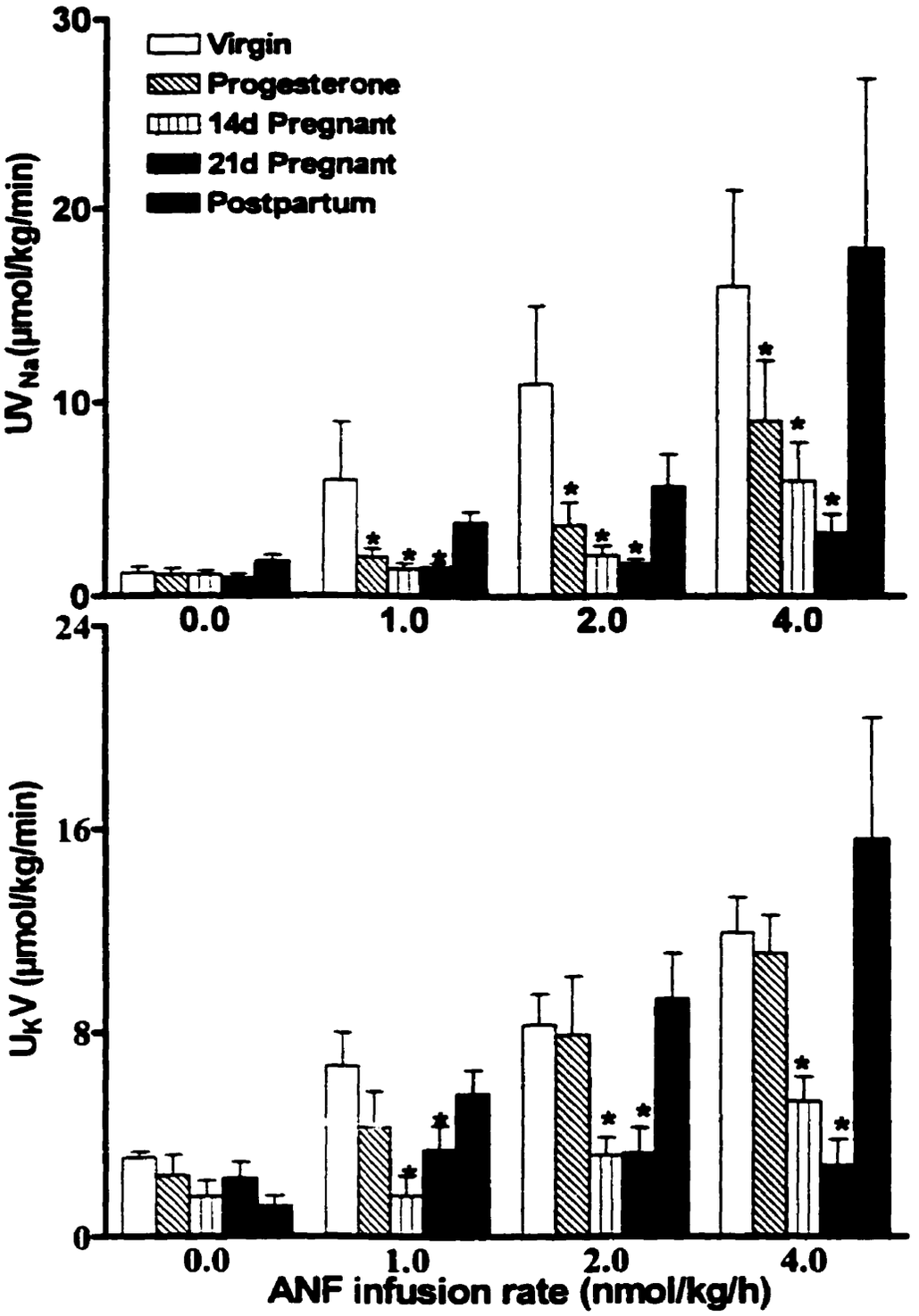

Virgin
Progesterone
14d Pregnant
21d Pregnant
Postpartum
UV_{Na} (µmol/kg/min)
30
20
10
0
0.0
1.0
2.0
4.0
U_KV (µmol/kg/min)
24
16
8
0
0.0
1.0
2.0
4.0
ANF infusion rate (nmol/kg/h)

Fig. 3.6

Fig. 3.6. Influence of constant infusion of increasing concentration of atrial natriuretic factor on urinary sodium ($U_{Na}V$) and potassium (U_KV).

Of virgin, progesterone treated (progesterone), days 13-15 (14d) and 21 day (21d) pregnant and 3 day postpartum rats under urethane anesthesia. Data are means ± SE of 6-10 experiments. * $P < 0.05$, different from values for virgin rats at indicated dose of ANF.

3.2.7 Effects of ANF infusion on urinary cGMP:

Infusion of ANF caused a dose-dependent increase in cGMP excretion in all groups of rats (virgin, pregnant, postpartum and progesterone-treated). However, there was no difference in the effects of ANF on urinary cGMP excretion among virgin, pregnant and progesterone-treated rats. On the other hand, at 2 and 4 nmol/kg/h ANF caused less excretion of cGMP in postpartum than in virgin rats (Table 3.2).

3.2.8 Modification of the renal effects of ANF by ANF_{4-23}.

Infusion of truncated analog of ANF, ANF_{4-23}, mimicked the effects of ANF on blood pressure and GFR and on urinary volume, sodium, potassium, and cGMP in both virgin and pregnant rats. However, unlike the renal effects of ANF, effects of ANF_{4-23} were greater in pregnant than virgin rats. For example, the increase in sodium excretion over basal following the infusion of ANF_{4-23} was 173 ± 40% in pregnant and only 67 ± 19% in virgin rats. ANF_{4-23} increased the effects of 1 nmol/kg/h ANF on GFR by 21% in virgin rats and by 62% in pregnant rats. The natriuretic and kaliuretic effects of concomitant infusion of ANF_{4-23} plus ANF on days 13-15 pregnant rats were not different from its effects in virgin rats (Table 3.3). However, the effect of ANF_{4-23} plus ANF on urine volume of virgin rats remained significantly higher than that in day 13-15 pregnant animals (Table 3.3).

3.2.9 Modification of the renal effects of ANF by thiorphan:

Like ANF_{4-23}, the endopeptidase inhibitor thiorphan also mimicked the renal and hemodynamic effects of ANF. Thiorphan did not produce a significant natriuretic effects but it produced significant effects on MBP, GFR, urinary volume, and potassium in virgin rats and on urinary volume and potassium in pregnant rats (Table 3.4). MBP, GFR, and urinary excretion of sodium and potassium in virgin and pregnant rats following the infusion of thiorphan plus ANF did not differ; however, the urine output was still less in pregnant than in virgin rats (Table 3.4).

TABLE 3.2. Effect of ANF infusion on urinary cGMP excretion in virgin, progesterone (prog)- treated, 13-15 days and 21 day pregnant and 3 day postpartum rats

Animals	ANF (nmol/kg/h)			
	0	1	2	4
			cGMP (pmol/min)	
Virgin	1.5 ± 0.54	17±3.7	50±10.7	102±15.4
Prog-treated	1.1± 0.29	20±4.1	45±8.5	71±6.
14d pregnant	1.2 ± 0.22	14±3.3	39±11.7	81±15.1
21d pregnant	1.62 ± 0.59	17±2.5	48±5.5	73±1.6
Postpartum	1.18±0. 22	15±4.5	27±3.7*	62±3.7*

Values are means ± SE for 5-6 experiments *different ($P < 0.05$) from virgin in the column.

Table 3.3 Effects of infusion of ANF_{4-23} plus ANF on MBP and renal function of virgin (V) and 13-15 day pregnant (P) rats

Variables	Animals	Basal	ANF_{4-23}	ANF_{4-23} plus ANF 1nmol/kg/h	ANF 2
MBP (mmHg)	V	117±3	106±3[a]	91±5[a,b]	91±5[a,b]
	P	106±3[c]	98±3	79±3[a,b]	90±6[a]
GFR (ml/kg/min)	V	3.6±0.18	5.9±0.45[a]	7.7±0.3[a,b]	15.4±3.5[a,b]
	P	5.1±0.36[c]	7.2±0.58[a,c]	9.1±0.8[a,c]	15.7±3.5[a,b]
V (µl/kg/min)	V	25±1.7	48±4.7[a]	123±8.4[a,b]	156±3[a,b]
	P	19±1.7[c]	36±3.1[a,c]	72±13.0[a,b,c]	86±14[a,b,c]
$U_{Na}V$ (µmol/kg/min)	V	1.3±0.3	2.1±0.4	5.9±0.2[a,b]	5.9±0.5[a,b]
	P	1.0±0.2	2.3±0.5	4.5±1.3[a]	8.6±3.1[a,b]
U_KV (µmol/kg/min)	V	2.1±0.3	4.1±0.7[a]	9.2±1.7[a,b]	15.0±4.9[a,b]
	P	2.6±0.4	4.8±1.2	5.8±0.9[a]	16.4±0.6[a,b]
$U_{cGMP}V$ (pmol/min)	V	1.4±0.5	3.7±0.8[a]	9.6±1.6[a,b]	not done
	P	0.8±0.2	2.8±0.6[a]	11±2.1[a,b]	not done

MBP - mean blood pressure; GFR - glomerular filtration rate; U - urinary; V - volume. Mean ± SE of 5-10 rats under urethane anesthesia. Dose of ANF_{4-23} was 25 nmol/kg by bolus injection followed by 36 nmol/kg/h by infusion. [a] Different ($p < 0.05$) from the basal values in the same row; [b] different ($p<0.05$) from the value after ANF_{4-23} in the same row; [c] Different ($p < 0.05$) from the immediate top value in the same column. Mean±SE, n= 5-10.

TABLE 3.4. Effects of infusion of thiorphan and of thiorphan plus ANF on MBP and renal function of virgin (V) and 13-15 day pregnant (P) rats

Variables	Animals	Basal	Thiorphan	Thiorphan+ANF
MBP (mmHg)	V	130±3	119±2[a]	102±2[a]
	P	110±4[b]	107±5	90±6[a]
GFR (ml/kg/min)	V	4.1±0.60	6.7±0.55[a]	10.6±0.6[a]
	P	5.7±0.49[b]	6.9±0.54	9.3±0.71[a]
V (µl/kg/min)	V	27±2.7	47±3.7[a]	120±8.7[a]
	P	19±2.1[b]	28±3.7[a,b]	53±11.1[a,b]
$U_{Na}V$ (µmol/kg/min)	V	1.5±0.1	2.1±0.4	3.7±0.3[a]
	P	1.1±0.3	1.7±0.5	3.2±0.7[a]
U_KV (µmol/kg/min)	V	2.2±0.6	4.5±0.9[a]	9.6±2.1[a]
	P	1.3±0.2	2.9±0.3[a]	5.8±0.9[a]

MBP - mean blood pressure; GFR - glomerular filtration rate; U - urinary; V - volume. Dose of thiorphan was of Mean±SE of 5-6 rats under urethane anesthesia. Thiorphan was infused at a rate of 24 µmol/kg/h for 30 min followed by thiorphan plus 1 nmol/kg/h ANF. The dose was [a] Different ($p < 0.05$) from the virgin in the same row; [b] different ($p < 0.05$) from the immediate top value for virgin rats in the same column.

3.3 Effects of bolus injections of ANF on urinary volume, sodium and cGMP excretion in rats under different hormonal states:

In the studies described so far, ANF was administered by intravenous infusion. Since it can not be determined whether this procedure simulates physiological changes in ANF levels, additional studies were done to determine the effects of bolus injections of ANF. The renal effects of ANF infusion and bolus injection were qualitatively similar. Therefore, bolus injections were also used to determine hormonal states which could simulate the influence of pregnancy on renal effects of ANF.

Bolus intravenous injections of ANF at doses of 0.01nmol/kg, 0.1 nmol/kg and 1.0 nmol/kg caused a dose-dependent increases in urinary volume, sodium and cGMP in all groups of rats. The maximal effect of ANF was observed within the first 10 minutes following its injection and urine volume as well as other variables returned to control levels by 30 minutes (Fig. 3.7). Data presented here are those obtained during the first 10 minutes after ANF injection. Quantitatively the effects of bolus injections of ANF in different groups (Fig. 3.8) were similar to effects of infusion (Tables 3.5 and 3.6).

The effects of ANF on urine and sodium excretion was significantly less in pregnant than in virgin rats (Table 3.5 and 3.6). Urinary volume in response to different doses of ANF in postpartum rats was comparable to values in virgin rats; however, the natriuretic effect of ANF was significantly greater in postpartum than in virgin rats (Table 3.6).

Treatment of virgin rats with progesterone caused a significant decrease in the effects of ANF on urine volume at all doses studied (Table 3.5).

Relative to OVX-estradiol-17β treated animals, the diuretic and natriuretic effects of ANF was significantly less in OVX-progesterone treated animals (Tables 3.5 and 3.6).

The natriuretic effects of ANF was significantly less in rats treated with a combination of estradio-17β plus progesterone relative to that in OVX rats treated with estrogen alone (Table 3.6).

Basal urinary cGMP was lowest in OVX rats and highest in postpartum animals (Table 3.7); likewise, ANF caused a significantly greater increase in cGMP excretion in postpartum rats than in virgin, gravid, and OVX rats.

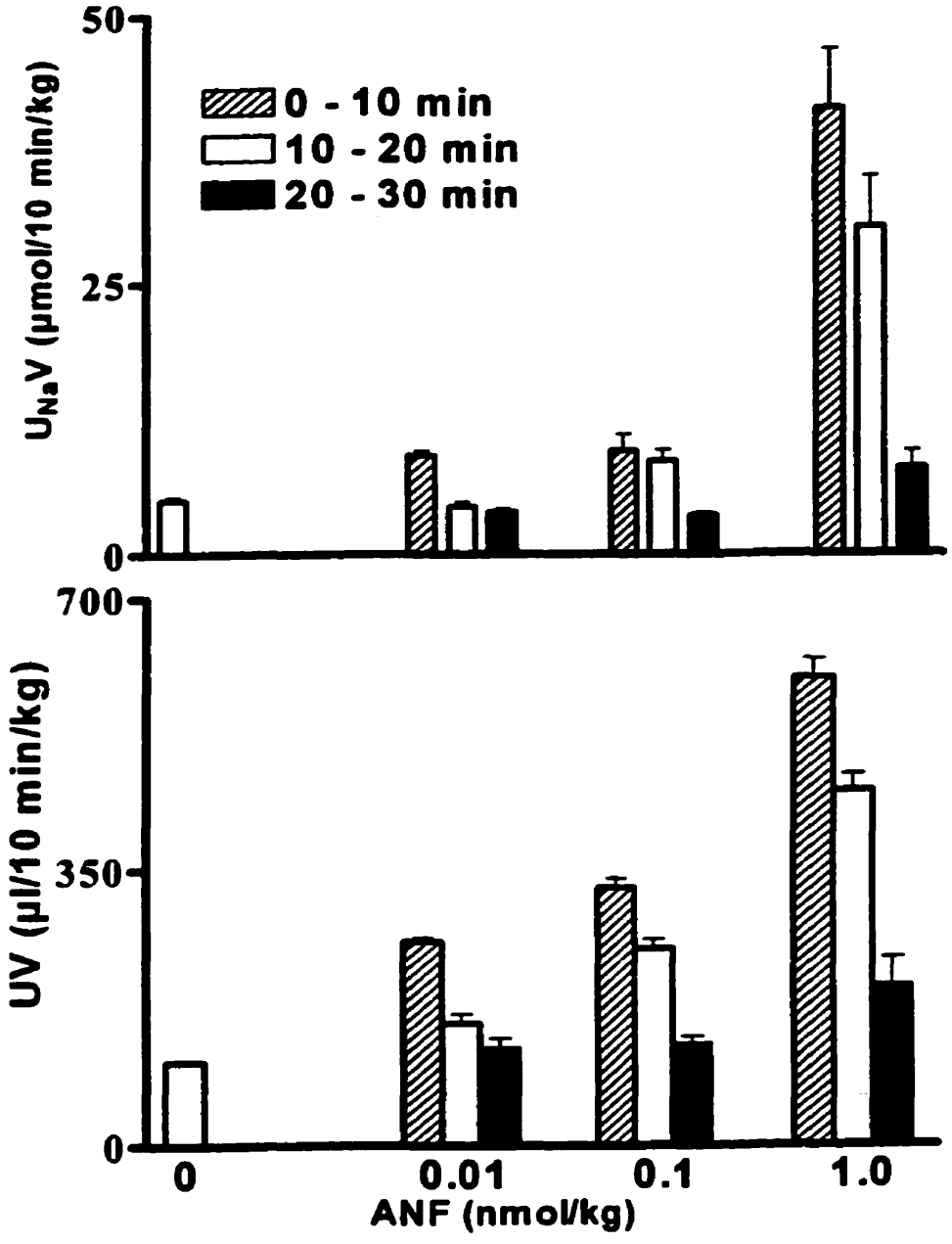


Fig. 3.7. Time course of the diuretic and natriuretic effects of bolus intravenous injection of ANF into virgin female rats under urethane anesthesia.

Means ± SE of 5 experiments.

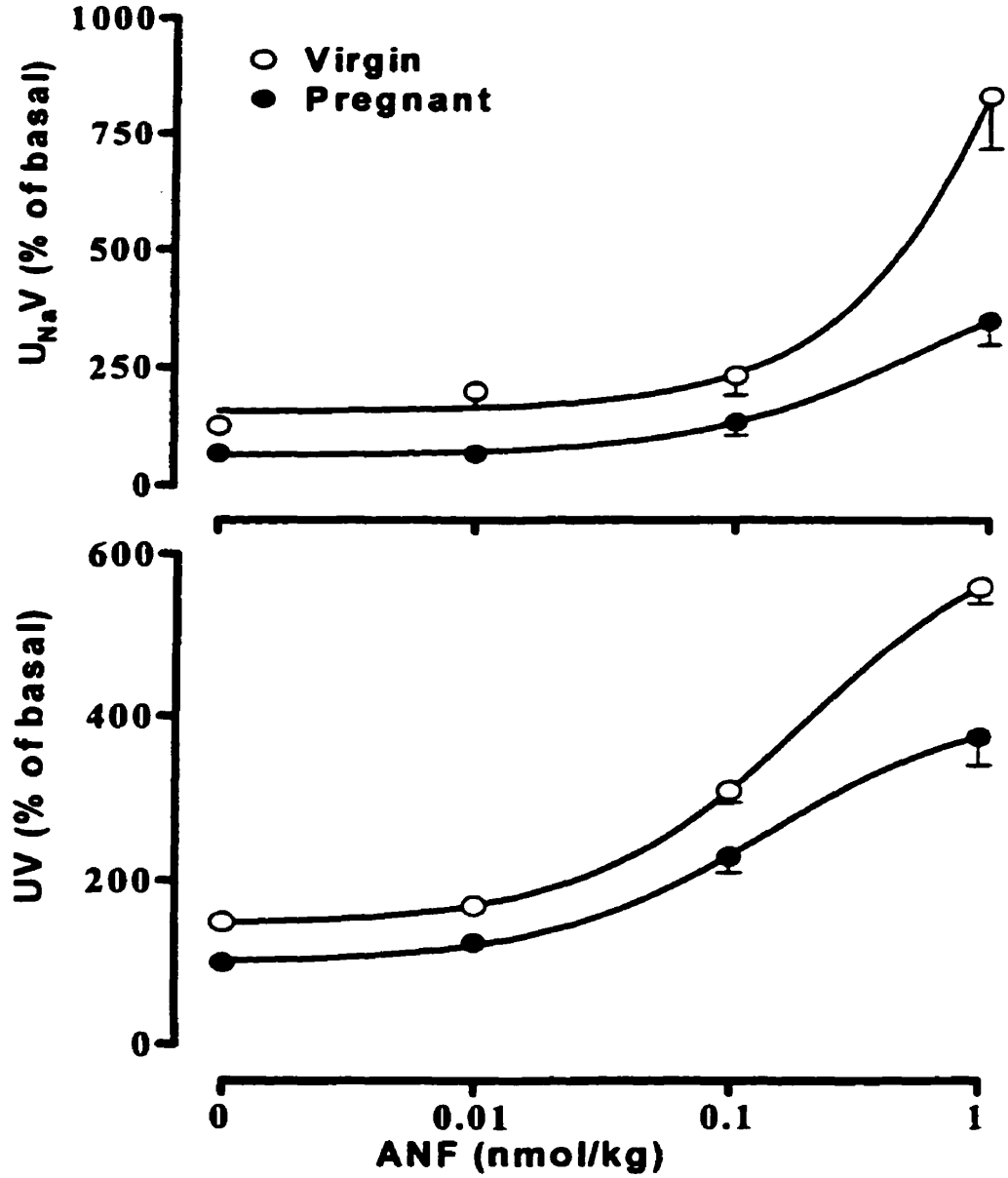


Fig. 3.8. Diuretic and natriuretic effects of ANF in virgin and 13-15 day pregnant rats under urethane anesthesia.

Responses during the first 10 min after bolus injections of ANF intravenously are shown as percent of basal values.

Table 3.5. Urine volume during the first 10 min after bolus injections of ANF in female rats under different hormonal states.

Animals	BW (g)	basal	0.01	ANF nmol/kg 0.1	1.0
	g			µl/10 min	
Virgin	219±4	107±4	254±10	326±11	594±20
Pregnant	331±21[a]	72±8[b]	91±12[b]	150±4[b]	260±13[b]
Postpartum	310±36[a]	91±8	163±8[b]	288±26	762±73[b]
Virgin+Prog.	230±5	89±5[b]	163±15[b]	256±26[b]	436±26[b]
OVX + Oil	240±6	110±3	253±6	349±13	542±21
OVX + Est.	236±10	102±2	263±8	384±11[b]	620±25
OVX + Prog.	225±9	97±2[c]	206±14[bc]	322±38	488±23[b]
OVX + Est+Prog.	217±8	105±6	213±8[d]	299±9[d]	583±31

Pregnant, 13-15 day; postpartum 72 h; OVX-ovariectomized 10-14 days earlier; Prog-progesterone (2 mg/kg/day x3); Est-estrogen (0.1 mg/kg/day x3). Significance ($P < 0.05$) in the same column compared as follows: a with all others; b with virgin; c with OVX-Oil; d with OVX-Est. Data are means ± SE of 5 experiments.

Table 3.6. Urinary sodium during the first 10 min after bolus injections of ANF in female rats under different hormonal states.

Animals	BW (g)	basal	0.01	ANF nmol/kg 0.1	1.0
	g			µmol/10 min	
Virgin	219±4	3.8±0.4	9.2±0.4	9.8±1.3	41.4±5.4
Pregnant	331±21[a]	2.5±0.4[b]	2.5±0.5[b]	4.6±0.5[b]	11.9±0.9[b]
Postpartum	310±36[a]	10.7±3[b]	22±4.6[b]	57±13.4[b]	131±23[b]
Virgin+Prog.	230±5	3.1±0.6	9.2±2.9	17.7±4.2[b]	35.2±3.8
OVX + Oil	240±6	5.0±0.4[b]	10.6±1.3	14.3±0.6[b]	16.6±2.2[b]
OVX + Est.	236±10	7.7±0.6[b]	26.5±5.3[c]	27.7±5.3[b]	45.3±17[c]
OVX + Prog.	225±9	6.9±1.[bd]	10.7±1.9[d]	10.4±2.6	16.9±4.2[d]
OVX + Est+Prog.	217±8	2.6±1.3	3.7±1.5[cd]	8.5±2.2[cd]	38±5.1

Pregnant, 13-15 day; postpartum 72 h; OVX-ovariectomized 10-14 days earlier; Prog-progesterone (2 mg/kg/day x3); Est-estrogen (0.1 mg/kg/day x3). Significance ($P < 0.05$) in the same column compared as follows: a with all others; b with virgin; c with OVX-Oil; d with OVX-Est. Data are means ± SE of 5 experiments.

Table 3.7. Urinary potassium during the first 10 min after bolus injections of ANF in female rats under different hormonal states.

Animals	BW (g)	basal	0.01	ANF nmol/kg 0.1	1.0
	G			µmol/10 min	
Virgin	219±4	5.9±0.8	13.3±2.3	22.7±3.6	25.2±4.1
Pregnant	331±21[a]	2.8±0.6[b]	3.1±1.3[b]	12.1±3.4[b]	17.1±5.7[b]
Postpartum	310±36[a]	13.2±2.3[b]	25±6.6[b]	45.8±9.8[b]	27.2±6.2
Virgin+Prog.	230±5	11.5±2.9[b]	12.2±1.9	30.4±8	42.5±12.1
OVX + Oil	240±6	6.3±0.6	14.3±1.0	18.8±1.1	27.5±5.7
OVX + Est.	236±10	7.7±0.6	26.5±5.3[bc]	27.7±5.3	42.1±3.9
OVX + Prog.	225±9	10.7±2.3[b]	14.4±2.6	15.6±2.8	27.6±6
OVX + Est+Prog.	217±8	7.1±.8	16±3.6[d]	21.9±4.5	35.2±6.1

Pregnant, 13-15 day; postpartum 72 h; OVX-ovariectomized 10-14 days earlier; Prog-progesterone (2 mg/kg/day x3); Est-estrogen (0.1 mg/kg/day x3). Significance ($P < 0.05$) in the same column compared as follows: a with all others; b with virgin; c with OVX-Oil; d with OVX-Est. Data are means ± SE of 5 experiments.

3.4 DISPOSITION OF ANF

3.4.1 ANF pharmacokinetics.

Plasma ANF as a function of time yielded a rapid phase of decline (half-life ~2.5 min) followed by a relatively slow phase of decline (Fig. 3.9). There was no significant difference in plasma half-life, volume of distribution, and plasma clearance of ANF between virgin and 20 day pregnant rats (Table 3.8).

3.4.2 Renal metabolism of ANF and NEP activity.

Metabolism of ANF by renal cortical membrane preparations from both virgin and 20 day pregnant rats was relatively rapid and did not differ (Fig. 3.10). Renal cortical membranes from virgin and gravid rats degraded ANF at an apparent rate (pmol/min/μg protein) of 45 ± 0.6 and 45 ± 0.5, respectively. The addition of 25 μM phosphoramidon to cortical membrane preparations completely inhibited the metabolism of ANF.

NEP activity in the cortical membranes from virgin and 20 day pregnant rats did not differ significantly (Fig. 3.11)

Table 3.8. Pharmacokinetics of ANF in virgin and 20 day pregnant rats under pentobarbital anesthesia

Variables	Virgin	Gravid
Body weight (g)	250±10	320±10*
Plasma half-life (min)	2.51±0.42	3.13±0.47
Plasma clearance (ml/kg/min)	115±19	124±26
Volume of distribution (ml/kg)	371±44	526±120

Values are means ± SE of 6 experiments; $p < 0.01$ compared with values for virgin rats.

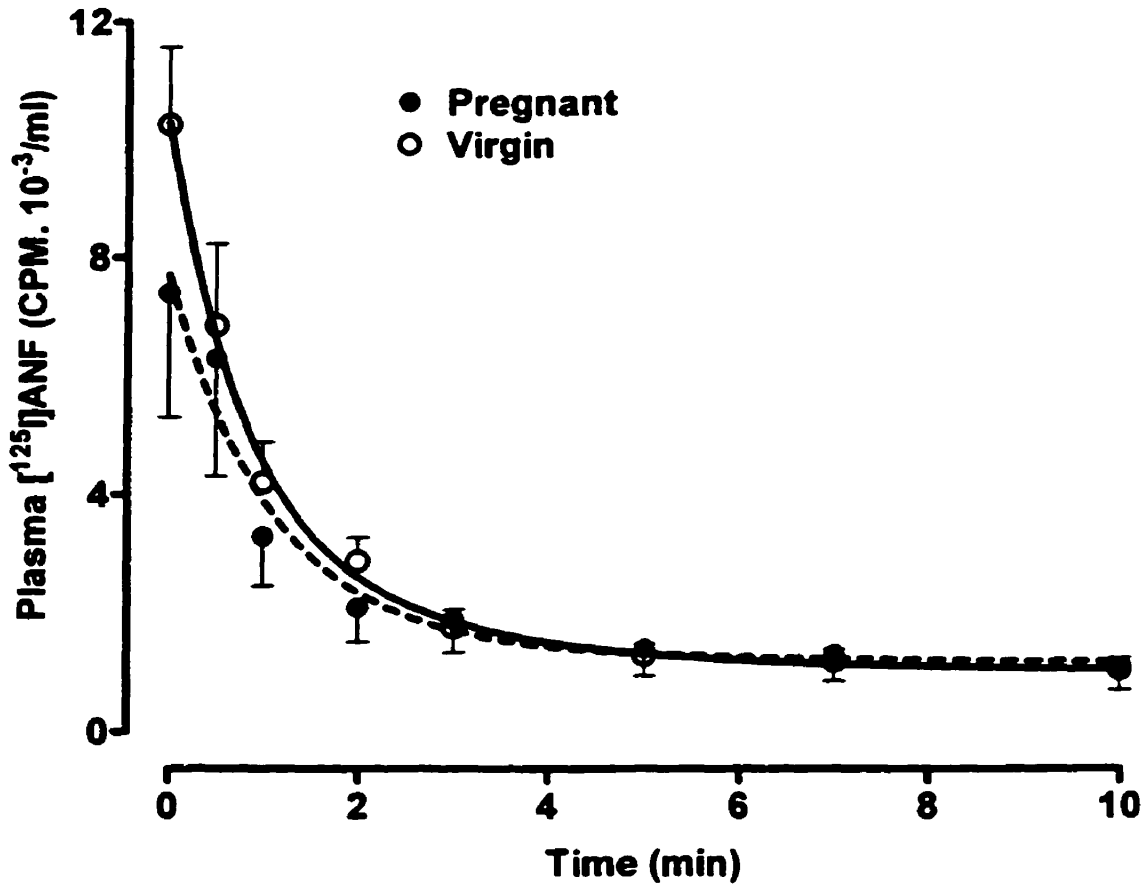


Fig. 3.9. Plasma atrial natriuretic factor (ANF) concentration as a function of time after a bolus intravenous injection of ^{125}I-ANF into pentobarbital-anesthetized virgin and 20 day pregnant rats.

Data are means ± SE of 6 experiments.

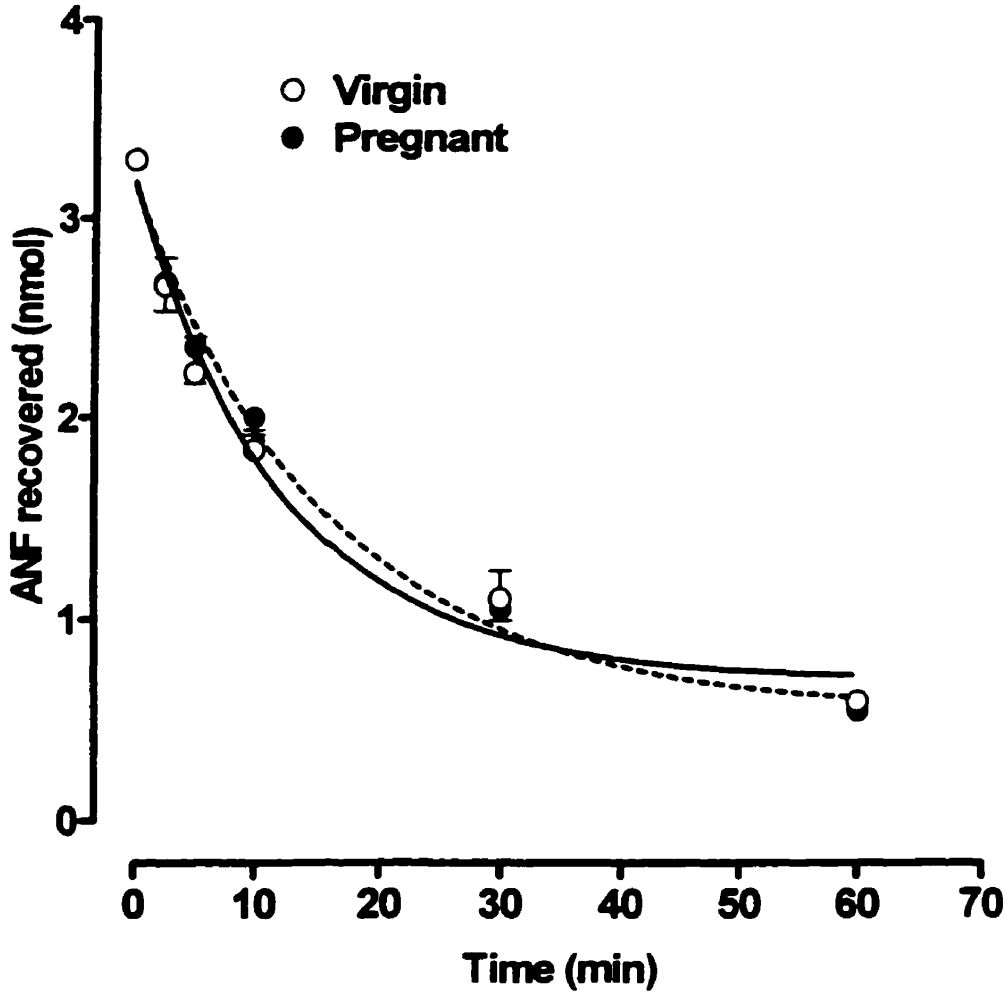


Fig. 3.10. Metabolism of ANF by renal cortical membrane preparations from virgin and 20 day pregnant rats.

Cortical membrane protein was incubated with 50 μM ANF at 37 °C; shown are unmetabolized ANF values. Data are means ± SE of 5 experiments.

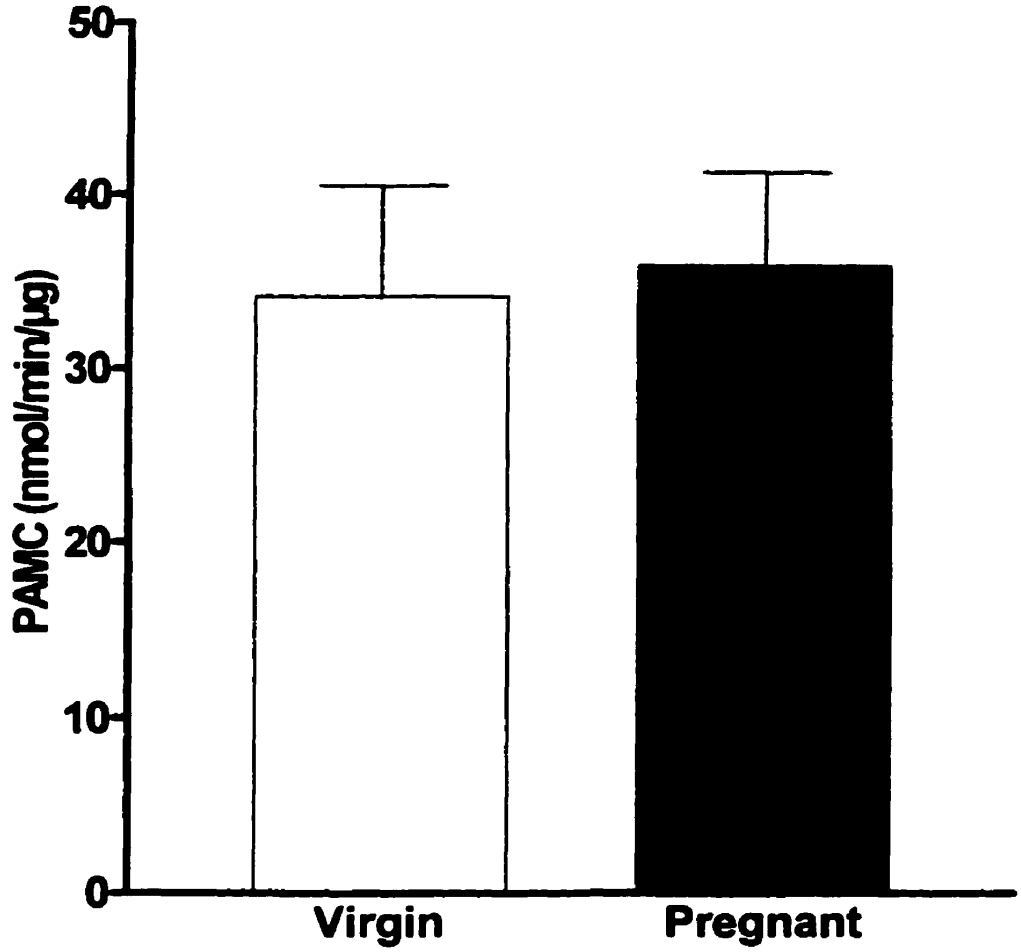


Fig. 3.11. Neutral endopeptidase activity in cortical membranes from virgin and 20 day pregnant rats.

Expressed as the rate of formation of Phe-amidomethyl-coumarin (PAMC) from N-succinyl-Ala-Ala-Phe-7-amido-4-methylcoumarin per minute per microgram of cortical membrane protein. Data are means ± SE of 7 experiments.

3.5 ANF RECEPTORS

3.5.1 ANF receptors in renal glomeruli.

The binding of ^{125}I-ANF$_{1-28}$ to glomerular membrane preparations from both virgin (Fig. 3.12a) and pregnant (Fig. 3.12b) rats was to a single site. ANF$_{1-28}$ completely displaced bound ^{125}I-ANF in preparations from both groups of animals. However, the truncated ligand ANF$_{4-23}$, which binds only to ANF-C receptors, displaced ^{125}I-ANF only partially from preparations from virgin but completely from preparations from pregnant rats. The B_{max} for ANF-GC receptors in glomeruli of virgin rats was significantly higher than in tissues from pregnant rats (Table 3.9). However, the maximum number of ANF-C receptors was higher in the glomeruli of pregnant than of virgin rats. Pregnancy did not significantly modify the apparent binding affinity of ANF to ANF-GC (Table 3.9) or ANF-C receptors.

3.5.2 ANF receptors in renal papillae

As in the case of glomeruli, the binding of ^{125}I-ANF to papillary membranes from virgin (Fig. 3.13a) and pregnant (Fig.3.13d) was to a single site. However, unlike the glomeruli, the truncated ligand ANF $_{4-23}$ did not displace ^{125}I-ANF from its binding sites in papillae (Fig. 1C and D). The B_{max} of ^{125}I-ANF binding to ANF-GC receptors in membranes of the papillae from virgin rats was significantly greater than that to pregnant rat preparations; binding affinity did not differ in the two groups of rats (Table 3.9).

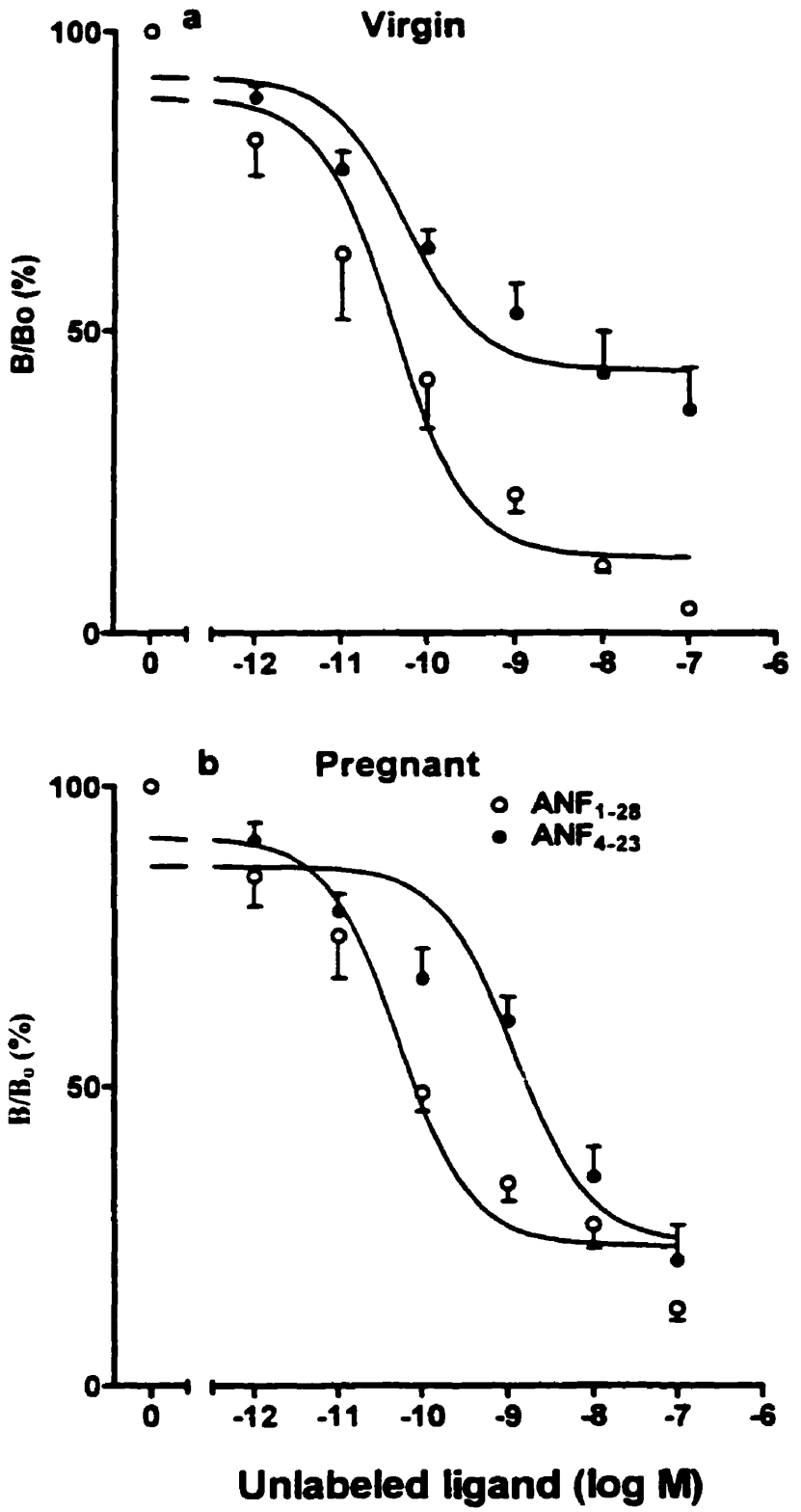

a
Virgin
100
50
0
B/Bo (%)
0
-12
-11
-10
-9
-8
-7
-6
b
Pregnant
ANF1-28
ANF4-23
B/Bo (%)
Unlabeled ligand (log M)

Fig. 3.12. Displacement curves of ^{125}I-labeled ANF by unlabeled ANF_{1-28} ANF_{4-23} in membranes of renal glomeruli.

From virgin (a) and 15-17 day pregnant (b). Membranes were prepared from 3-4 rats for each experiment and incubated with 10 pM ^{125}I-ANF_{1-28} for 2 h at room temperature in the presence of increasing concentrations of unlabeled ANF_{1-28} or ANF_{4-23}. B and B_0 represent, respectively, specific binding in the presence and the absence of unlabeled ligands. Data are means ± SE of 5 separate experiments, each in duplicate.

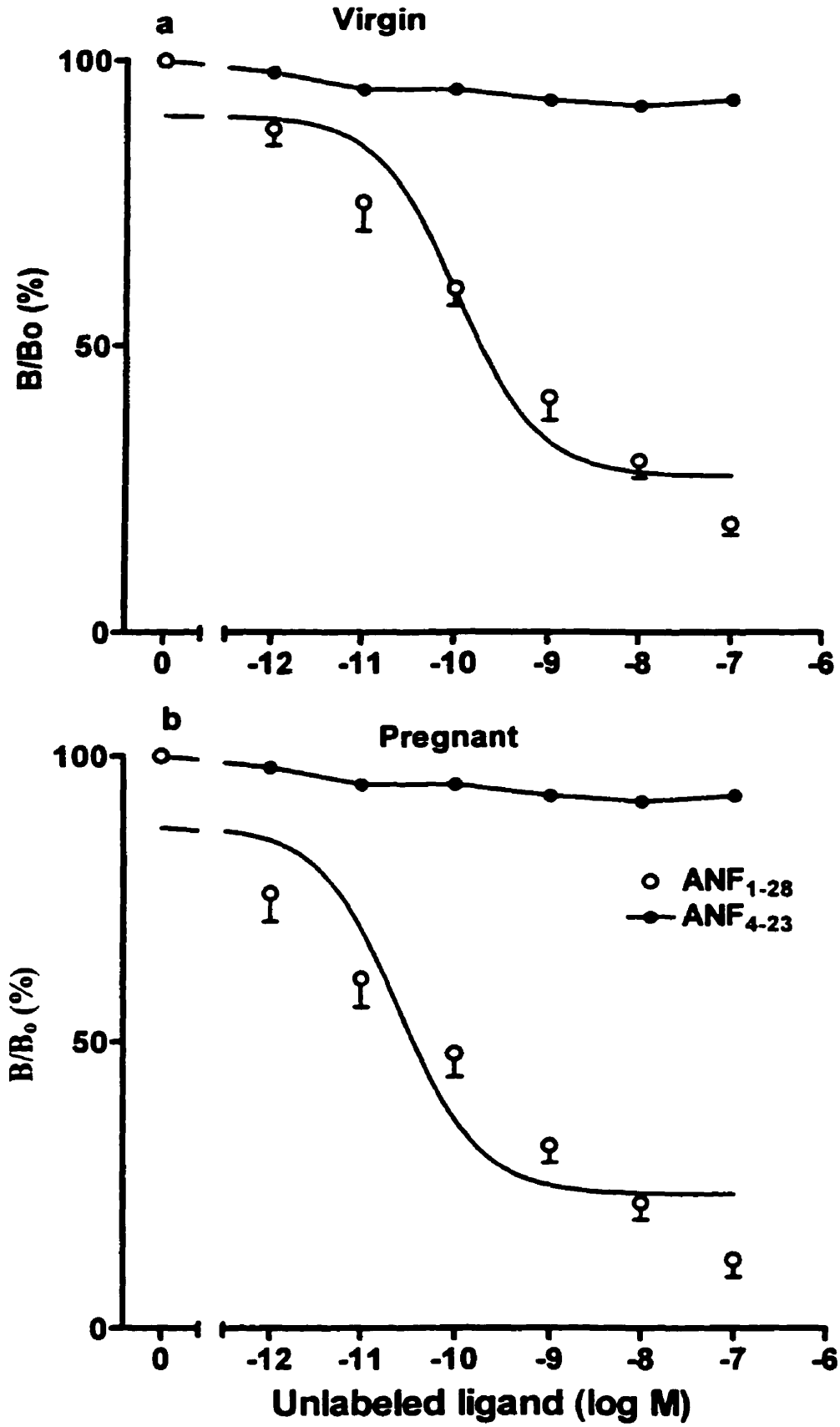

a
Virgin
100
50
0
B/Bo (%)
0
-12
-11
-10
-9
-8
-7
-6
b
Pregnant
100
50
0
B/Bo (%)
ANF1-28
ANF4-23
0
-12
-11
-10
-9
-8
-7
-6
Unlabeled ligand (log M)

Fig. 3.13. Displacement curves of ^{125}I-labeled ANF by unlabeled ANF_{1-28} ANF_{4-23} in membranes of renal papillae.

From virgin (a) and 15-17 day pregnant (b). Membranes were prepared from 3-4 rats for each experiment and incubated with 10 pM ^{125}I-ANF_{1-28} for 2 h at room temperature in the presence of increasing concentrations of unlabeled ANF_{1-28} or ANF_{4-23}. B and B_o represent, respectively, specific binding in the presence and the absence of unlabeled ligands. Data are means ± SE of 5 separate experiments, each in duplicate.

Table 3.9. ANF receptors in glomeruli and papillae from virgin and 15-17 day pregnant rats

Tissue	ANF-GC	ANF-GC	ANF-C	ANF-C	ANF-C
	K_D (pM)	B_{max} (fmol/mg protein)	B_{max}	B_{max} (% of total)	K_D (pM)
Glomeruli (V)	76±26	132±28	139±20	57±8	79±11
Glomeruli (G)	138±47	36±17*	95±10	79±7*	61±13
Papillae (V)	169±35	120±12	0	0	
Papillae (G)	138±30	57±7*	0	0	

Membranes were incubated with 10 pM [^{125}I]ANF_{1-28} in the presence of 0 to 0.1 μM unlabeled ANF_{1-28} or its truncated analog ANF_{4-23} at room temperature for 2 h. The B_{max} derived from the displacement curves using ANF_{1-28} minus the B_{max} for ANF-C receptors derived from displacement curves using ANF_{4-23} yielded B_{max} for ANF-GC receptors. Data are means ± S.E. of five separate experiments, each in duplicate; * different ($p < 0.05$) from the corresponding value for the virgin rats. V, virgin; G, 15-17 day pregnant.

3.5.3 cGMP production by renal glomeruli.

The basal cGMP production rates (pmol/ min/mg protein) by glomeruli of virgin and pregnant rats did not differ and were 1.28 ± 0.18 and 0.96 ± 0.16, respectively. ANF caused a concentration dependent increase in cGMP production in glomeruli from both virgin and pregnant rats; however, the effect of ANF was significantly less in pregnant than in virgin rats (Fig. 3.14a); also, the maximal increase in cGMP formation (pmol/min/mg protein) was 6.62 ± 0.69 in virgin rats and significantly ($P < 0.005$) greater than that (4.27 ± 0.89) in pregnant rats.

3.5.4 cGMP production by renal papillae.

The basal as well as ANF-stimulated cGMP production by renal papillary cell suspensions was lower than that by glomeruli in both virgin and pregnant animals. The basal cGMP production (pmol/min/mg protein) was 0.17 ± 0.03 by papillae from virgin rats and 0.19 ± 0.07 by tissues from pregnant rats; these values did not differ from each other. As in the case of glomeruli, ANF caused a significantly less increase in cGMP formation by papilla from pregnant than from virgin rats (Fig. 3.14b). The maximal ANF-induced cGMP generation (pmol/min/mg protein) was 1.9 ± 0.07 in virgin rats and was significantly greater than that (1.1 ± 0.04) in pregnant animals.

The basal as well as ANF (100 nM) stimulated cGMP production by papillae from virgin and postpartum rats did not differ from each other (Fig. 3.15).

3.5.5 Medullary cGMP production:

Basal as well as ANF-stimulated cGMP generation in inner medullary collecting duct cells was ~10-fold greater than in outer medullary collecting duct cells. ANF caused a concentration-dependent increase in cGMP accumulation in both cell types. However, there was no significant difference in the effects of ANF on cGMP production by tissues from virgin and pregnant rats (Table 3.10)

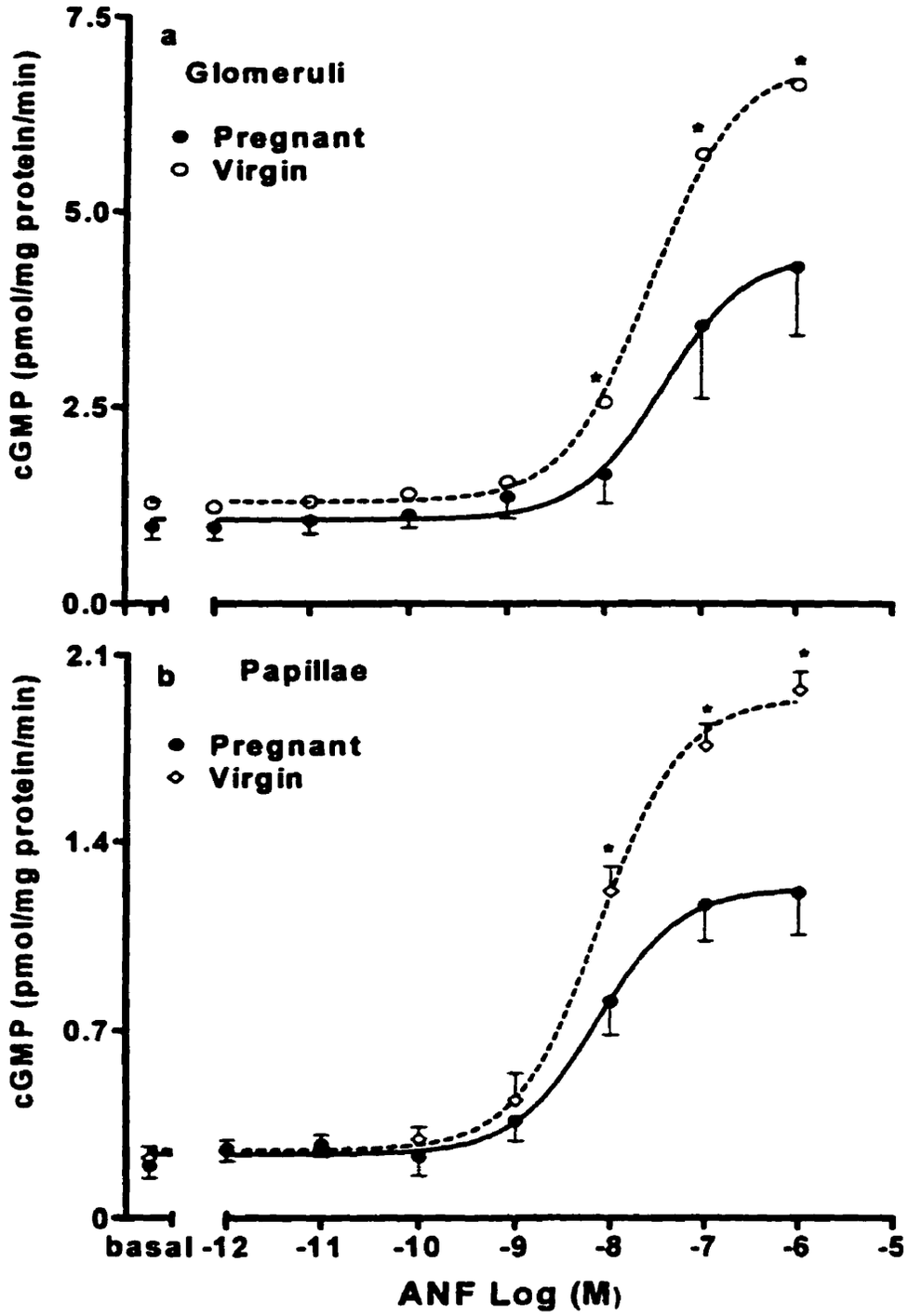

a
Glomeruli
Pregnant
Virgin
cGMP (pmol/mg protein/min)
7.5
5.0
2.5
0.0
b
Papillae
Pregnant
Virgin
cGMP (pmol/mg protein/min)
2.1
1.4
0.7
0
basal
-12
-11
-10
-9
-8
-7
-6
-5
ANF Log (M)

Fig. 3.14. Effect of ANF on cGMP production by the glomeruli (a) and papillae (b).

From virgin and 15-17 day pregnant rats. Cell suspensions of tissues from 3-4 animals for each experiment were preincubated with 1 mM 3-isobutyl-1-methylxanthine at 37 °C for 10 min and then incubated for 10 min with increasing concentrations of ANF. The reaction was stopped by the addition of acidic ethanol, and cGMP in the supernatant was quantified by RIA. Values are means ± SE of 5 separate experiments each in duplicate. $^{*}P < 0.05$, different from corresponding values for pregnant rats.

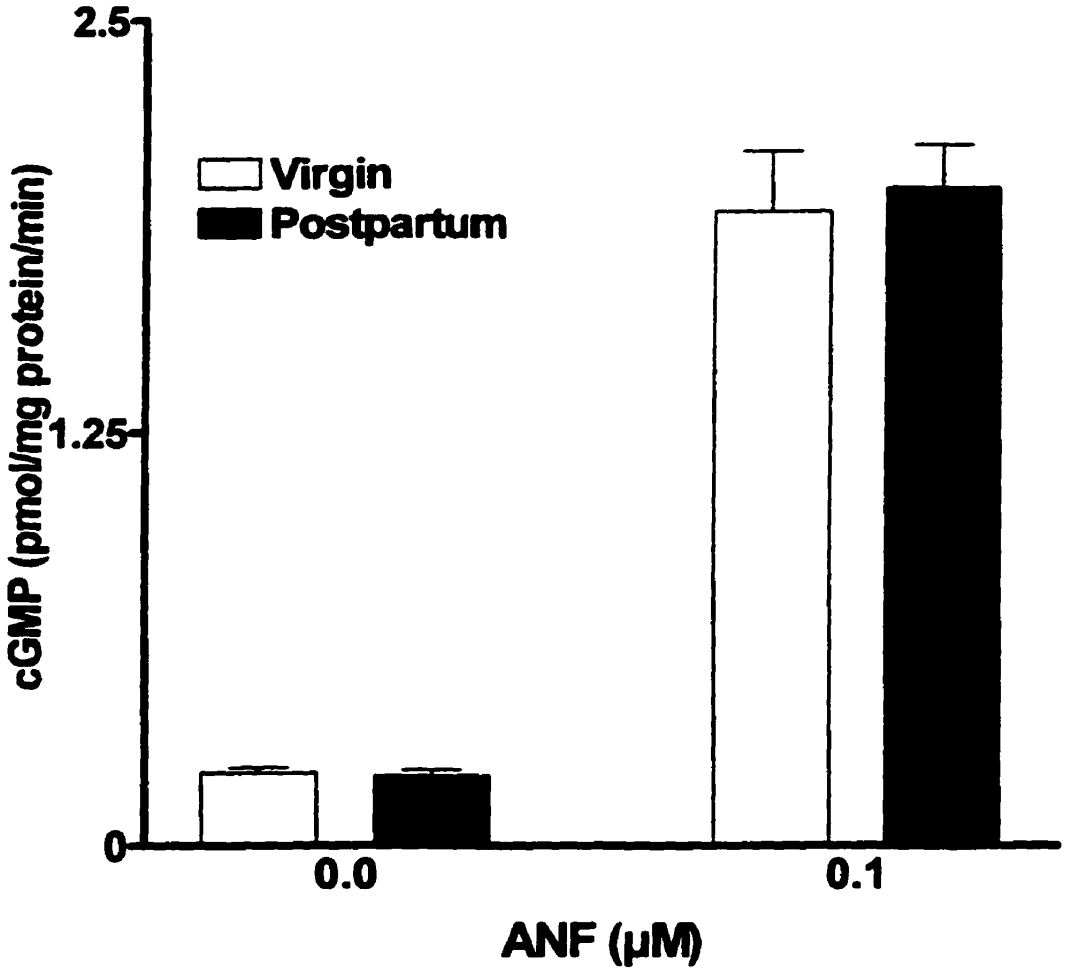


Fig. 3.15. Effect of ANF on cGMP production by papillae from virgin and postpartum rats.

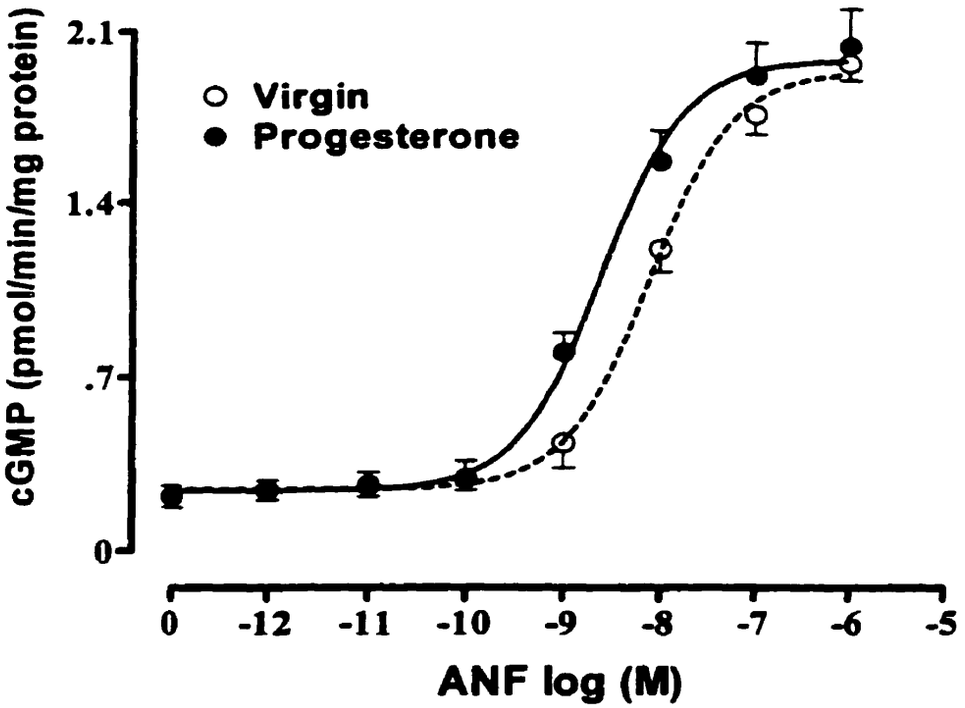
Four separate experiments were done, each with cell suspensions of papillae from one virgin and one *day-2* postpartum rat to study the effect of a single concentration (100 nM) of ANF; cGMP was determined as described in fig. 3.14. Data are means ± SE of 4 separate experiments, each in duplicate.

TABLE 3.10 Effect of ANF on cGMP formation by inner and outer medullary collecting duct cells from virgin and 13-15 day pregnant rats

ANF (nM)	V IMCD	G IMCD	V OMCD	G OMCD
		cGMP (fmol/mg protein)		
0	4712±1004	3185±651	403±110*	494±27*
0.1	5168±1040	5970±1539	461±78*	531±13*
1.0	5297±1048	6369±1689	602±109*	632±43*
10	7168±940	7765±218	576±165*	642±34*
100	8870±464	12870±4761	838±114*	683±100*

Values are means ±SE of 4-5 experiments, each in duplicate IMCD; inner medullary collecting duct cells; OMCD, outer medullary collecting duct cells; * Different ($P < 0.05$) from values in IMCD.

Fig. 3.16 Effects of ANF on cGMP production by renal papillae in virgin and progesterone treated rats.



3.5.6 ANF receptor mRNAs in renal papillae

The riboneuclease protection assays detected mRNA for all the three ANF receptors (GC-A, GC-B, and C) in the papilla of both virgin and pregnant rats (Fig. 3.17). The mRNA levels for GC-A receptors (Fig. 3.17, A and D) and ANF-C receptors (Fig. 3.17, C and F) were significantly lower in the papillae from pregnant than in papillae from virgin rats. Pregnancy did not lead to a change in GC-B receptor mRNA levels (Fig. 3.17, B and E).

3.5.7 ANF GC-A and GC-B receptor proteins in renal Papillae.

There was a clear expression of ANF GC-A receptor protein in the papillae both under reducing and nonreducing conditions. The signal for GC-A protein was less in the tissues of pregnant than of virgin rats in all three separate experiments as illustrated in fig. 3.18, top; no obvious difference in GC-A protein in the lungs (used as controls) of virgin and pregnant rats was noted.

GC-B protein was barely detectable in the papillae in any of the three experiments performed, although its presence was obvious in the uterus (used as a positive control).

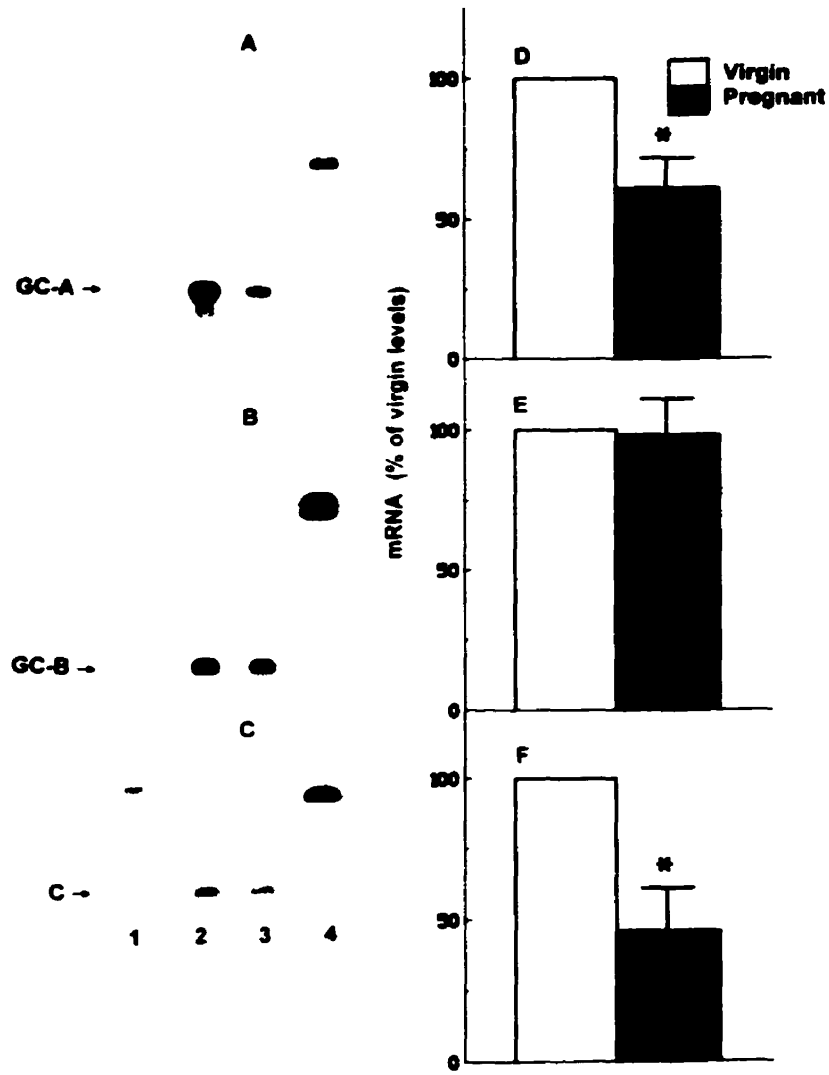


Fig. 3.17. mRNA levels of ANF guanylyl cyclase (GC)-A, GC-B and C receptors. In the papillae of virgin and pregnant rats. A-C: *lane 1,* tRNA control; lane 2, mRNA for GC-A (A), GC-B (B) and C receptors (C) in virgin rats; lane 3, in 15-17 day pregnant rats; lane 4, antisense RNA probe for GC-A (A), GC-B (B) and C receptors (C). D-F; histograms show relative densities of mRNAs in papillae of pregnant animals as a percent of virgin control for GC-A (D), GC-B (E) and C receptors (F). Data are means ± SE of 4 separate experiments. *P < 0.05, different from corresponding virgin levels.

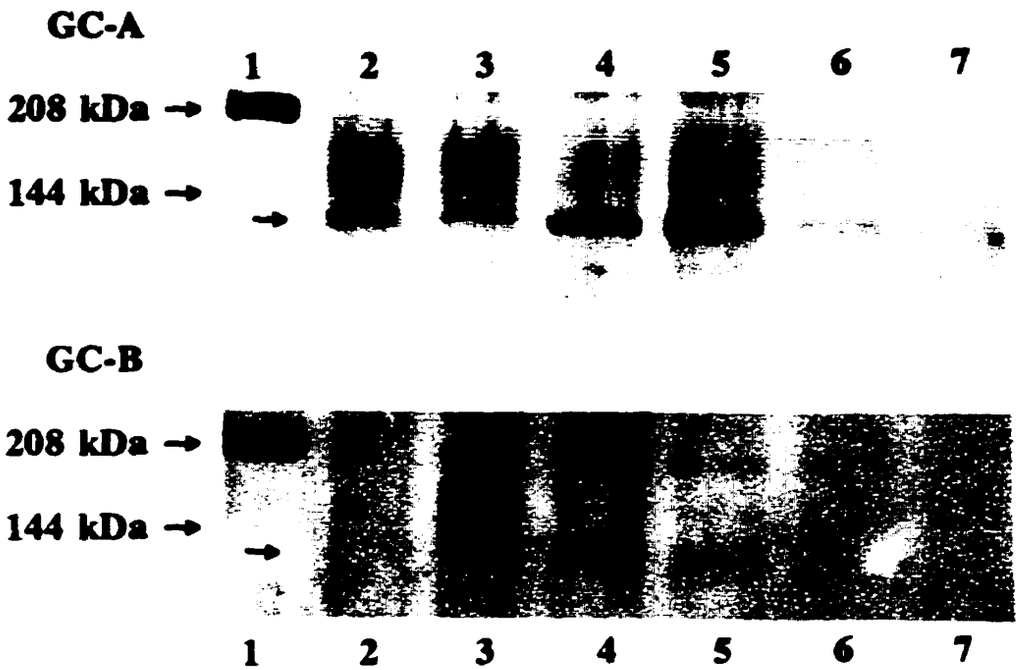


Fig. 3.18. Western blots of ANF GC-A (top) and GC-B (bottom) receptors in the papillae of virgin and 15-17-day pregnant rats.

In top (GC-A) and bottom (GC-B): lane 1, ladder; lane 2, papillae from virgin rats; lane 3, papillae from pregnant rats. Top: lane 4 and 5, lungs from virgin and pregnant rats, respectively. Bottom: lane 4 and 5, uteri from virgin and pregnant rats, respectively. Lane 2-5 are under reducing conditions. Lanes 6 and 7 are papillae, respectively, from pregnant and virgin rats under nonreducing conditions. Western blots were prepared utilizing polyclonal anti-receptor antibodies.

3.6 SUMMARY

- Pregnancy attenuated the diuretic and natriuretic effects of ANF; these effects returned to virgin levels during the postpartum period.
- ANF pharmacokinetics and renal metabolism were not altered during pregnancy.
- Pregnancy was associated with a significant decrease in ANF-GC-A receptors in renal glomeruli and papillae as determined by radioligand binding. Pregnancy led to a decrease in ANF-GC-A receptor mRNA and receptor protein in renal papillae; these studies were not done with renal glomeruli.
- Pregnancy was associated with a decrease in cGMP production by renal glomeruli and papillae.
- The influence of pregnancy on the renal effects of ANF was partially but not completely mimicked by progesterone.

CHAPTER FOUR: DISCUSSION

Pregnancy is associated with marked changes in fluid and electrolyte homeostasis; in addition to marked changes in hormone balance and cardiovascular adaptations, there is progressive expansion of the ECF and plasma volumes, and an increase in GFR and RPF (28). The mechanisms contributing to these changes in fluid and electrolyte balance are largely unknown, although marked adaptations must occur in the regulatory systems of the body in order for both fluid expansion and net sodium retention to take place.

The production of renin and its substrate angiotensinogen is markedly elevated during gestation; this increase in plasma renin activity is associated with high ANG II and aldosterone secretion (28, 34, 67); in addition pregnancy is accompanied by high levels of prostaglandins which have been shown to stimulate renin release and contribute to the already high circulating aldosterone (70). Since aldosterone promotes sodium reabsorption by the renal collecting ducts, the increased aldosterone levels are conducive to the plasma volume expansion observed during pregnancy.

Marked changes also occur in the ADH and thirst mechanisms during pregnancy (43, 44, 83). ADH increases water permeability of the collecting ducts and thereby promotes water retention. Plasma ADH levels are not altered during pregnancy and in both pregnant women and animals there is an increased water and salt appetite despite a significant fall in plasma osmolality (44). Pregnancy is associated with lowering of the threshold for ADH release; these changes in the osmoregulatory system during pregnancy favor fluid expansion.

In the face of an expanding extracellular fluid space one expects changes in the regulatory mechanisms that promote solute and water excretion within the body. ANF by virtue of its diuretic, natriuretic, aldosterone suppressant activity acts as a counter-regulatory of the renin-ANG II-aldosterone-axis. Normally expansion of the ECFV is accompanied by elevation in plasma ANF levels and low plasma aldosterone. However, this antagonistic relationship between plasma aldosterone and ANF levels is not observed during pregnancy as plasma ANF levels remain unaltered throughout the course of gestation (316, 318, 322). In contrast, plasma ANF levels rise and aldosterone levels fall in the immediate postpartum period (318, 320) accounting for the diuresis and natriuresis seen in the puerperium in order to restore the expanded maternal volume to normal.

Therefore it seems that there is some mechanism that prevents ANF from exerting its usual biological effects during pregnancy, a possibility that is conducive to the fluid expansion observed during gestation. Therefore, these studies tested the hypothesis:

An attenuation of the renal effects of ANF might be required to allow physiologically needed plasma volume expansion during pregnancy.

Therefore, the objectives of this study were to determine if the renal effects of ANF are attenuated during pregnancy.

For this purpose virgin, pregnant and postpartum rats were used. Since data revealed that pregnancy did attenuate the renal effects of ANF, studies were done to determine the underlying mechanisms. For this purpose we determined the influence of

pregnancy on ANF pharmacokinetics and renal metabolism, on ANF receptors and on the effects of ANF on cGMP production by renal tissues.

The rat has been extensively used to study the changes seen during pregnancy as well as to understand the mechanisms that are responsible for the adaptations in fluid and electrolyte homeostasis associated with pregnancy (22, 26, 33). Most of the information about changes on renal hemodynamics, renal tubular function, changes in the osmoregulatory system as well as many other adaptations seen during pregnancy has been derived from studies performed in the pregnant rat (12, 17, 43, 44).

Thus the pregnant rat seemed a suitable model to conduct our studies on the renal effects of ANF during pregnancy.

Like in humans, rat pregnancy is associated with an increase in extracellular fluid and plasma volume, an increase in RPF, GFR cardiac output and a decrease in blood pressure. The decrease in blood pressure is more pronounced near term and GFR tends to reach a peak near mid-pregnancy and then decline to prepregnant state by the end of gestation (10). The present study also found an increase in plasma volume, inulin space (which represents the ECFV) and GFR and a decrease in blood pressure. Most of these variables returned to prepregnant values in the postpartum period (Table 3.1). These findings are essentially in agreement with those reported in the literature (5, 10). However, the present study recorded relatively low basal GFR and plasma volume and a significant decrease in blood pressure near midgestation. It is very likely that certain discrepancies between our findings and those of others (5, 10) may be caused by

difference in the experimental conditions of these studies; for example, our data are derived from urethane-anesthetized rats.

One possible mechanism by which fluid and electrolyte expansion during pregnancy might not be opposed by ANF could be a decrease in the synthesis and release of ANF. To test this possibility we studied the changes in plasma ANF and aldosterone along the course of pregnancy by serial measurements before, during and after pregnancy (Fig. 3.1). Significant increase was observed in plasma aldosterone throughout the course of pregnancy and a precipitous drop after parturition. On the other hand, plasma ANF levels during pregnancy remained unchanged except for a slight but significant increase on day six of pregnancy. Plasma ANF levels were significantly elevated in the immediate postpartum period. The findings in plasma ANF are in agreement with those reported by Kaufman *et al.* (336) who demonstrated that atrial stretch-induced ANF release was significantly higher in day seven pregnant than in virgin control rats. Over all, these data suggest that fluid expansion during pregnancy is not contributed by a decrease in plasma ANF. The increase in aldosterone, while conducive to plasma volume expansion, could also suggest that the usual aldosterone-suppressant effect of ANF is not fully operative during pregnancy; this inference is consistent with the previously reported decrease in the aldosterone-suppressant effects of ANF on zona glomerulosa cells from pregnant rats (324a).

Although plasma ANF levels during pregnancy do not change in the direction as required for fluid expansion, the sudden increase in plasma ANF during the postpartum may be of physiological relevance. The increase in ANF and the fall in aldosterone

during the immediate postpartum period could contribute to the diuresis and natriuresis seen in the puerperium and the restoration of the expanded maternal volume to normal. It is quite possible that the decrease in aldosterone during the postpartum period is contributed by restoration of or an increase in the aldosterone-suppressant effects of ANF for which there is experimental evidence (324a).

We should mention that the serial plasma ANF values reported in these study are higher than those reported in the literature (318, 322); this is probably due to our method of collection of the blood samples. We collected blood from the tail vein under ether anesthesia which has been shown to be associated with elevated plasma ANF levels (337). Nevertheless, the values of this study follow the general trend that has been observed (322). Given the wide variations in plasma ANF between different animals, the serial samples from the same animal perhaps are fair indication of changing profile of ANF levels during pregnancy and postpartum.

The puzzle of increased activity of the renin-ANG II-aldosterone in the face of an expanding plasma volume has received a lot of attention and led to some controversy. Some workers (41) have hypothesized that the regulatory systems sense the intravascular volume as an underfilled state, thus stimulating the renin-ANG II-aldosterone axis. The peripheral vasodilatation that occurs early in pregnancy is perhaps due to elevated levels of vasodilators such as PGI_2, NO and the decreased responsiveness of the vascular system to pressor hormones (70, 75, 90); in addition the placenta might act as an arteriovenous fistula leading to a further decrease in the peripheral vascular resistance. Therefore, the effective blood volume, a measure of how the arterial volume fills the vascular space,

would fall; this would be sensed by the baroreceptors in the carotid sinus and aortic arch as well as the volume sensors in the atria and venous circulation and in turn lead to an activation of the sympathetic nervous system and stimulation of the renin-ANG-II-aldosterone system.

However, plasma levels of norepinephrine, an index of sympathetic activity, are not elevated during pregnancy (338, 339), as one would expect if the vascular system was under filled. Thus, the underfill theory seem to be too simplistic to explain the changes in fluid and electrolyte homeostasis that is observed during gestation.

Other workers (340) have suggested that the increased aldosterone levels during pregnancy exceeds the physiologic requirements and do not contribute to the sodium retention of pregnancy; this view is based on the fact that the mineralocorticoid-induced sodium retention ceases after the body achieves a new steady state despite continued mineralocorticoid administration, a phenomenon termed aldosterone escape (340, 341). However, aldosterone secretion changes appropriately in response to various stimuli such as sodium intake, saline infusion, upright posture and diuretic therapy (71, 72, 73) during pregnancy; it is thus very unlikely that aldosterone levels during pregnancy are beyond the physiological requirements with little or no role in the sodium retention associated with pregnancy.

In the context of the physiology of aldosterone levels during pregnancy, the data of this study at least demonstrates that its increase during pregnancy and decrease during the postpartum period is in accordance with the changes in plasma volume.

As mentioned earlier we did not observe changes in plasma ANF levels during the course of rat pregnancy although atrial natriuretic factor levels in plasma are thought to be an index of atrial pressure. Given the substantial increase in plasma volume during pregnancy one would expect ANF levels to be elevated. However, ANF levels not only remain unchanged during the course of pregnancy but also ANF secretion in response to atrial stretch are blunted during pregnancy (336).

There may be several reasons which prevent a change in ANF levels during gestation. First, pregnancy is associated with reduced vascular tone and an expanded maternal vascular compartment which leads to a decrease in the effective circulating volume and therefore to a reduction in right atrial pressure; however, similar degrees of atrial stretch was associated with a blunted ANF release in pregnant rats (336) which implies the involvement of other factors besides the reduced filling pressure in the lack of change in plasma ANF levels. Such a mechanism which prevents change in ANF levels during pregnancy could be the resetting of the atrial volume sensors to accommodate the expanding plasma volume. Second, pregnancy could result in an increased degradation of ANF; however, this possibility was ruled out in this and other studies (342). A third possibility could be that chronic states of volume expansion such as during pregnancy might not lead to elevated ANF levels as observed during acute increases in plasma volume; this notion is supported by the findings of Nadel *et al.* (318), who demonstrated that ANF levels remained unaltered in pregnant and non pregnant uninephrectomized rats treated with DOCA and high salt diet, a condition that is known to expand the extracellular space.

There are numerous studies on the renal effects of ANF. However, there are very few studies that have examined the influence of pregnancy on these effects (343 344, 345). In the present study we found that the natriuretic and diuretic effect of ANF were significantly less in pregnant than in virgin rats. In fact this attenuation of the renal effects of ANF increased as pregnancy progressed. For example, the diuretic and natriuretic effects of ANF was less in 21 day than in 13-15 day pregnant rats (Fig. 3.5; 3.6); this is consistent with the observations that more than 50% of the increase in ECFV in rats is seen in the last week of gestation and maximal refractoriness to the renal effects of ANF at this time would ensure the ideal plasma volume expansion to be achieved.

Our data that pregnancy attenuated the renal effects of ANF are in conformity with the findings of other investigators, who also found a significant decrease in the natriuretic effects of ANF in pregnant rat (343, 344) and goats (345). Kaufman at. el. also found that atrial stretch of rats at various stages of pregnancy was not accompanied by significant diuresis and natriuresis as was observed in virgin rats (346). A decrease in the natriuretic effects of a bolus injection of atriopeptin II has also been reported although these workers attributed this to the change in the volume of distribution of atriopeptin II (319). In the present study we used continuous infusion (to achieve steady state plasma ANF levels) rather than a bolus injection; and we found that there was no difference in irANF before or after exogenous ANF infusion. Thus, the reduced responsiveness to ANF during pregnancy cannot be attributed to differences in plasma ANF levels.

The finding that irANF were similar between virgin and pregnant rats suggests that factors other than ANF probably play an important role in the increased GFR and

decreased vascular tone observed in pregnancy. Recent studies have implicated increased endothelium derived relaxing factor (NO) for the changes in GFR and RPF during pregnancy (90, 91).

The decrease in the diuretic and natriuretic effects of ANF in pregnancy found in this study is unlikely to be due to difference in its effects on MBP of virgin and pregnant rats. Although the basal MBP was less in pregnant rats, the hypotensive effect of ANF was relatively less in pregnant than in virgin rats, in conformity with other reports (343) such that the MBP of virgin and pregnant rats at different doses of ANF did not significantly differ. This is further evidence for the attenuation of the effects of ANF during pregnancy at several sites; its renal as well as its hypotensive effect.

The major mechanism by which ANF inhibits diuresis and natriuresis is by inhibiting sodium reabsorbtion in the inner medullary collecting ducts (347) and to a certain extent by increasing the filtered load of solute i.e. GFR (256); the contribution of other factors, such as changes in renal plasma flow, transepithelial driving forces, inhibition of renin, and interactions with other hormones might be less important (119, 277).

We did not find any significant difference in the absolute GFR at different doses of ANF between virgin and pregnant rats (Fig. 3.4a). However, the increment in GFR was significantly less in pregnant than in virgin rats. ANF increases GFR by causing dilatation of the afferent arterioles and contraction of the efferent arterioles; it also increases the surface area available for filtration by rising k_f, the ultrafiltration coefficient, by its direct action on the mesangium (254, 255). The finding that ANF caused less increment in GFR

than in virgin rats implies that the actions of ANF at the glomeruli are blunted during pregnancy; the contribution of this to the attenuation of the diuretic and natriuretic effects of ANF can not be excluded, although ANF can produce diuresis and natriuresis independent of changes in GFR (257, 348). It is also possible that the smaller increase in GFR following ANF infusion in pregnant than in virgin rats could be due to a higher basal GFR in the former than in the latter group of animals.

The excessive increases in GFR, particularly in virgin and postpartum rats, at higher doses of ANF, found in this study might also reflect washout of residual inulin during profuse diuresis rather than the true changes in GFR.

Most of the effects of ANF, including on the glomeruli (265) and collecting ducts, are mediated by activation of particulate guanylyl cyclase and the generation of cGMP (265, 273). Therefore, urinary levels of cGMP were measured to find out if the attenuation of the renal effects of ANF are accompanied by decreased urinary cGMP levels. In the present study, however, basal urinary cGMP excretion of virgin and pregnant rats did not significantly differ (Table 3.2). It has been reported that in unrestrained Long-Evan's rats, the 24-h urinary excretion of cGMP is increased during pregnancy, presumably due to increased nitric oxide formation (90). The discrepancy between our data and that of the above mentioned study (90) is perhaps due to the relatively short period of urine collection, physical restraints caused by the anesthetic and so on.

There was no significant decrease in urinary cGMP excretion in pregnant rats despite a decrease in the natriuretic effects of ANF. Moreover, the natriuretic effects of

ANF in postpartum rats was comparable to that of virgin rats although its effect on cGMP excretion was less in postpartum than in virgin rats. The likely explanation for a lack of difference in the effect of ANF on urinary cGMP in different groups of rats (virgin, pregnant and postpartum) could because urinary cGMP is derived from various tissues, which might mask any attenuation in the effects of ANF on specific renal sites.

The over all effects of any hormone are detemined not only by its actions on specific functional receptors, but also by its bioavilability. The studies involving concomitant infusion of ANF_{4-23} plus ANF and of ANF plus thiorphan were undertaken to shed some light regarding the mechanisms underlying the attenuation of the diuretic and natriuretic effects of ANF by pregnancy. ANF is degraded by two independent mechanisms; ANF receptor-C mediated internalization followed by lysosomal hydrolysis (231) and enzymatic degradation by neutral endopeptidases (326).

In the present study, intravenous infusion of ANF_{4-23}, a specific ligand for ANF clearance receptors and lacking direct (200) action increased fluid and solute excretion in both virgin and pregnant rats (Table 3.3); these data are in agreement with other studies which showed that truncated ANF-analogs induced increased diuresis and natriuresis and raised irANF levels in anesthetized rats (200). However, there was difference in the effects of ANF_{4-23} as well as its influence on the effects of ANF in virgin and pregnant rats. For example, ANF_{4-23} caused 67 ± 19% increase in sodium excretion in virgin rats whereas the increase in pregnant rats was 173 ± 40% . At equivalent rates of infusion of ANF_{4-23}, its natriuretic effect relative to that of ANF (1 nmol/kg/h) was ~37% in virgin rats and 133% in pregnant rats. Also ANF_{4-23} caused a greater potentiation of the effects

of ANF on GFR of pregnant than in virgin rats. Thus, the disproportionately greater potentiation of the effects of ANF by ANF_{4-23} in pregnant rats led to comparable natriuretic responses in pregnant and virgin rats (Table 3.3) and implied that pregnancy might lead to an increase in ANF-C receptors and in turn to the receptor-mediated metabolism of ANF; this possibility was not ruled out in these *in vivo* studies as we did not establish whether or not we used sufficient ANF_{4-23} to saturate all the clearance receptors. It should, however, be pointed out that ANF_{4-23} caused a greater potentiation of the effects of ANF on sodium excretion than on GFR, suggesting that sites other than the glomeruli such as collecting ducts might make important contribution in the modulation of the effects of ANF during pregnancy.

An inhibition of endopeptidase activity is also known to potentiate the effects of ANF; the administration of these inhibitors have been associated with increased diuresis, natriuresis and increased circulating ANF levels (246, 349). The present study also found that thiorphan, an endopeptidases inhibitor (249), produced an increase in fluid and sodium excretion. Although these effects were less pronounced than with ANF_{4-23}, the concomitant infusion of thiorphan plus ANF produced comparable natriuresis in virgin and pregnant rats (Table 3.4).

Thus, the simultaneous administration of ANF with inhibitors of its receptors or endopeptidase-mediated degradation, increased the natriuretic effect of ANF in pregnant rats to levels in virgin rats. In view of comparable post-infusion plasma ANF levels in virgin and pregnant rats it appears that ANF_{4-23} and thiorphan mainly inhibited the intrarenal degradation of ANF, as suggested by other studies (349). The findings with

ANF_{4-23} and thiorphan suggest that the attenuation of the renal effects of ANF in gravid animals was at least in part caused by increased metabolism. Consequently, studies were carried out to determine the influence of pregnancy on the disposition of ANF. Our findings suggest that changes in the disposition of ANF might play a minor role, if any at all, in the pregnancy associated attenuation of the diuretic and natriuretic effects of ANF.

ANF pharmacokinetics were determined by using tracer amounts of [^{125}I]-ANF as mentioned in the methods section; the pharmacokinetics of [^{125}I]-ANF has been reported to be identical to that of endogenous ANF_{1-28} (229). A rapid followed by a relatively slow decline in plasma ANF after a bolus injection as found in this study, has also been reported by several workers in rats (229 , 244, 245, 326), dogs (325), and sheep (350). This plasma pharmacokinetic profile of ANF perhaps reflects its receptor-mediated and receptor-independent metabolism (229, 246, 248). Despite the overall similarity, the plasma half-life of ANF determined in this study is slightly longer than that reported by others (229, 243, 244, 326) but identical with one report in rats (245). Probably this discrepancy reflects difference in techniques used to separate intact [^{125}I]-ANF, we used HPLC for separation while others (229, 326) used trichloroacetic acid precipitation. Because the plasma half-life is inversely related to the plasma clearance, the factors resulting in a somewhat shorter half-life would explain the slightly larger plasma clearance rates reported by these workers (229, 243, 326).

We are not aware of any studies on complete pharmacokinetics of ANF in pregnant rats. Data on plasma clearance of ANF determined in this study are very similar to values reported from continuous infusion of ANF in conscious virgin and pregnant rats

(342). Moreover, similar to the previously cited study (342), we did not find any significant difference in the plasma clearance rates between virgin and pregnant rats. A lack of effect of pregnancy on the plasma half-life of ANF has also been reported in sheep (350). Taken together, these observations suggest that the primary mechanism of the attenuation of the diuretic and natriuretic effects of ANF in pregnancy are not changes in its plasma pharmacokinetics.

The results of studies on the *in vitro* metabolism of ANF by renal cortical membrane and renal cortical NEP activity, were consistent with a lack of significant influence of pregnancy on plasma pharmacokinetics of ANF. The brush border of the proximal convoluted tubules in the renal cortex are rich with neutral endopeptidases and there is strong evidence that NEP of the proximal tubular brush border is responsible for the major receptor-independent metabolism of ANF (249, 351); the findings of this study supports this view. The significance of renal metabolism of ANF in its renal effects is supported by the observations that inhibitors of neutral endopeptidase increase plasma ANF levels (351) as well as the renal effects of ANF as found in the present and other studies (246). It can thus be assumed that an increase in endopeptidase activity during pregnancy could theoretically lead to a decrease in the biological effects of ANF. However, the present study found no difference in the metabolism of ANF by cortical membranes from virgin and gravid rats (Fig. 2). Because the NEP inhibitor phosphoramidon (328) almost completely inhibited the degradation of ANF by cortical membrane preparations, it can be assumed that ANF degradation was mediated by NEP. In addition to this, a direct measurement of NEP activity in renal cortical membranes from virgin and gravid rats again revealed no difference. Thus, our findings suggest that

the modulation of the renal effects of ANF during pregnancy cannot be contributed by changes in its NEP-mediated renal metabolism, although the possibility of changes in receptor-mediated metabolism of ANF can not be ruled out.

ANF induces diuresis and natriuresis by increasing the filtered solute load and by inhibiting sodium reabsorbtion in the inner medullary collecting ducts. The increase in the filtered load at the glomerular level is mediated not only by preglomerular dilatation and postglomerular constriction (253), but also, by an action on the glomerular mesangium, which contain ANF receptors and respond to ANF by marked cGMP production (255, 265). However, ANF can cause diuresis and natriuresis without increasing GFR (257, 258). The main site of action of ANF is thought to be the inner medullary collecting ducts where it inhibits sodium reabsorbtion (119, 273, 347); at this site ANF interacts with particulate *guanylyl cyclase* stimulating cGMP production which then inhibits amiloride-sensitive sodium channels either directly or through the activation of cGMP-dependent protein kinase (271, 272).

We found that ANF caused a smaller increase in GFR in pregnant than in virgin rats; this would suggest that pregnancy might modify effects of ANF both on the glomeruli and collecting ducts. Since we and others (342) did not find any changes in the metabolism of ANF during pregnancy, the possibility that a decrease in the diuretic and natriuretic effects of ANF during pregnancy might be caused by a downregulation of renal ANF-GC receptors was investigated. Our findings support this possibility.

Several investigators have shown that the rat glomeruli contain both ANF-GC and ANF-C receptors; in contrast, the rat papillae contain only ANF-GC linked receptors but

not ANF-C receptors (199, 216, 241, 331). There is also evidence from cross-linking and radioligand binding studies (199, 241, 331) that the renal papillae contain primarily GC-A linked ANF receptors. We also found that ANF_{4-23} a selective ligand for ANF-C receptors (200) displaced $[^{125}I]ANF_{1-28}$ binding from glomerular but not from papillary membrane preparations, suggesting that ANF-C receptors were not present in the papillae.

We measured the displacement of $[^{125}I]ANF_{128}$ by unlabeled ANF_{1-28} to quantify all the three ANF receptors and by ANF_{4-23} to estimate ANF-C receptors. Consistent with other studies (199, 216,241, 331) which demonstrated that the selective C-receptor ligand ANF_{4-23} was unable to displace $[^{125}I]ANF_{1-28}$ from papillae; we also found that ANF_{4-23} did not displace labeled ANF_{1-28} from papillary preparations, indicating the absence of ANF-C receptors in this tissue. However, ANF_{4-23} did displace $[^{125}I]ANF_{1-28}$ from glomerular membrane preparations, indicating the presence of ANF-C receptors in the glomeruli as reported by other workers (216, 241, 331). It is noteworthy to mention that studies have demonstrated that the majority of ANF receptors in the glomeruli are clearance receptors (200, 212). Similarly, we found that ANF_{4-23} displaced ~60% and 80% of the receptors in glomeruli in virgin and pregnant rats, respectively, confirming previous reports regarding the predominance of ANF-C receptor in the glomeruli.

Data of our study indicate a significant decrease in ANF-GC receptors and a relative increase in ANF-C receptors in glomeruli of pregnant rats (Table 3.9); this would suggest that an increase in receptor-mediated metabolism of ANF during pregnancy might contribute to the attenuation of its renal effects; this inference is in agreement with our *in vivo* studies, which revealed that ANF_{4-23} produced greater diuresis and natriuresis and a

greater potentiation of the diuretic and natriuretic effects of ANF in pregnant than in virgin rats (Table 3.3).

This study also demonstrates that the downregulation of ANF GC-A receptor density during pregnancy is associated with a decrease in the effects of ANF on cGMP production both in the glomeruli and papillae. We did not detect any difference in the effects of ANF on cGMP generations when we used preparations comprising of both outer and inner medullary tissues; however, we found that pregnancy caused a significant decrease in cGMP in response to ANF when we used the clearly defined papillae. It is reasonable to assume that using the entire medulla, which contain both outer and inner portions of collecting ducts, masked the difference in the effects of ANF on cGMP production by preparations from virgin and pregnant rats. It is very possible that the differences might be more pronounced in the papillae that contain the terminal collecting ducts than in the preceding portions of the nephron; the terminal collecting ducts contain maximal intensity of GC-A mRNA and ANF produces the maximal increase in cGMP in this portion (240, 331). In any case, a decrease in the maximal effects of ANF on cGMP generation by glomeruli and papillae of pregnant rats can explain the reported attenuation of the renal effects of ANF during gestation (333, 343, 344, 346).

Furthermore, we found comparable effects of ANF on cGMP production by papillae from virgin and postpartum rats; these findings are consistent with our *in vivo* observations that the diuretic and natriuretic effects of ANF are restored in the postpartum period.

To further confirm our data derived from binding studies, we quantified ANF receptor mRNAs by RNase protection assay and receptor proteins by Western blot analysis.

mRNAs for all the three ANF receptors (GC-A, GC-B and C) were detected in the rat papillae (Fig. 3.17); these findings are in conformity with previous reports documenting the expression of GC-A (240, 242) and GC-B (240) mRNA in the rat papillae. To our knowledge the expression of ANF-C receptor mRNA in rat papillae has not been previously examined, although the presence of ANF-C receptor mRNA has been demonstrated in pig papillae (230).

Western blot analysis clearly showed the presence of GC-A receptor protein in the papillae (fig. 3.18). However, GC-B-receptor proteins were barely detectable in the papillae but their expression was evident in the uterus which was used as a positive control (Fig. 3.18). An earlier study also found no GC-B in rat renal papillae (241). Thus, it seems that the principal form of GC-coupled ANF receptors in rat papillae is of the GC-A type. To our knowledge this is the first study to definitively establish the absence of GC-B receptor proteins in the renal papillae of the rat using Western blot analysis; others (199, 241) have arrived at a similar conclusion using either receptor cross-linking studies or by determining the ability of CNP (a specific ligand for GC-B) to stimulate cGMP production in the papillae.

We did not detect ANF-C receptors in the papillae by radioligand binding studies, which is in agreement with earlier studies reporting the absence of this receptor in the papillae (199, 216, 241).

A decrease in mRNA for GC-A receptors during pregnancy was accompanied by a decrease in receptor proteins. The specificity of the changes in ANF GC-A-receptor proteins in renal papillae during pregnancy is suggested by a lack of change in its expression in lungs.

The modulation of GC-receptors by pregnancy as our findings indicate primarily reflects changes in GC-A linked ANF receptors. This inference is supported by the data that GC-A receptor protein was much more abundant in papillae from virgin than from pregnant rats. Assuming that GC-B receptors are expressed in rat papillae the fact that we did not observe any decrease in GC-B receptor mRNA during pregnancy is suggestive of no or little change in receptor proteins. Moreover, ANF_{1-28} has a low affinity for GC-B receptors (208, 211, 213); it is thus seems reasonable to infer that the decrease in the effects of ANF_{1-28} on cGMP production on the papillae is primarily due to a downregulation of ANF GC-A receptors.

It is also noteworthy that similar to the findings in the papillae and glomeruli, we found that GC-A receptor protein in the adrenal zona glomerulosa cells (352) and GC-B receptor proteins in the uterus (352a) were downregulated during pregnancy. Since downregulation of ANF-GC in adrenals and uteri was associated with a decrease in the effects of ANF in these tissues (352, 352a), it is tempting to suggest there exists a causal relationship between an attenuation of the renal effects of ANF and a downregulation of its receptors.

Downregulation of ANF receptors has been reported in conditions associated with hypervolemia, such as congestive heart failure, renal failure, cirrhosis and DOCA-salt

induced hypertension (353, 354, 355). However, these conditions are also associated with increased plasma irANF and it has been suggested that the rise in irANF may be responsible for receptor downregulation (356). Hirata *et al.* (356) have demonstrated that exposure of vascular ANF receptors to ANF at physiological concentrations leads to a marked decrease in total ANF receptors with no change in affinity, suggesting that ANF downregulates its receptors probably due to receptor internalization and hydrolysis following ligand occupation.

Fluid expansion during pregnancy, on the other hand, is physiological and is not associated with an increase in irANF. Hence a downregulation of ANF receptors could not be attributed to changes in ANF levels. It is tempting to suggest that downregulation of ANF receptors during pregnancy might be a physiological mechanism by which the body achieves expansion of ECFV during gestation. In agreement with this assumption is the fact that we did not find global downregulation of ANF receptors but a selective regulation such that the significant changes in GC-A receptors were found in the tissues that are intimately involved in the maintenance of fluid and electrolyte homeostasis, that is in the renal glomeruli and papillae (this study) as well as in the adrenal gland (352).

Although our data clearly demonstrates that pregnancy is associated with a downregulation of ANF-GC receptors the physiological mechanism involved in this process is not clear from our studies. Several possibilities exist and we explored some such as the role of sex steroid hormones.

ANG II has also been shown to directly down regulate glomerular ANF receptors (357). Given the increased activity of the renin-angiotensin system during pregnancy it is reasonable to speculate that ANG II might be involved in the pregnancy-associated downregulation of ANF receptors. It has been demonstrated that renal denervation leads to up-regulation of ANF receptors in the glomeruli and ANG II can abolish this effect (358, 359). Our data do not rule out a role for ANG II in the downregulation of ANF receptors during pregnancy.

Studies (360, 361) have implicated increased renal sympathetic nerve activity for attenuating the natriuretic effects of ANF in cirrhotic rats since renal denervation restored the response to ANF. On the other hand, renal denervation was not accompanied with increased natriuresis in nephrotic rats (362). These pathophysiologic states of fluid expansion are usually accompanied with movement of intravascular fluid into the interstitial space leading to edema and ascites; the pregnancy associated fluid expansion dose not occur by similar mechanisms. Nevertheless, the possibility that renal sympathetic nerve activity might contribute to the modulation of the renal effects of ANF during pregnancy is an area that deserves exploration.

There are profound hormonal changes that occur during pregnancy which could lead to a downregulation of ANF receptors. Most noticeable, is the marked increase in plasma progesterone levels. Progesterone treatment is also known to mimic some of the changes observed during rat gestation: such as increases in plasma volume, and GFR (18). We have previously shown that progesterone inhibited the tocolytic (324b, 363), and aldosterone suppressant activity of ANF (324a, 332). This antagonistic relationship

between progesterone and ANF my be important in the relative refractoriness to the natriuretic and aldosterone suppressant activity of ANF (324a, 332), which may in part account for the physiological changes in body fluid and renal sodium handling during pregnancy and postpartum. There is evidence for a modulation of ANF receptors by sex hormones (332). We have demonstrated that estrogen upregulates and progesterone downregulates ANF receptors in the uterus and adrenal gland (332, 364).

Therefore, the principal objective of the experiments done under different hormonal states was to explore the mechanisms and the hormones responsible for modulating the diuretic and natriuretic effects of ANF during pregnancy. We tested if progesterone contributed to the pregnancy-associated refractoriness to the renal effects of ANF for several reasons. First, pregnancy is associated with a progressive increase in plasma progesterone, which has been shown to reproduce the effects of pregnancy on GFR and ECFV. Second, we have shown that progesterone attenuated the aldosterone-suppressant and tocolytic effects of ANF (324a, 324b).

Several groups of female rats under various hormonal states were used to find out if the influence of pregnancy was exerted via progesterone. The findings of this study do not clearly establish this relationship, although they do not exclude the possibility that progesterone might be a contributing factor. For example the diuretic effect of ANF was consistently less in virgin rats treated with progesterone than in untreated virgin rats (Table 3.5); likewise, injections of progesterone plus estrogen resulted in a decreased diuretic and natriuretic effects of ANF relative to its effects in OVX rats treated with estrogen alone (Table 3.5; 3.6). On the other hand, injection of progesterone to virgin rats

or to OVX-estradiol treated rats did not decrease the natriuretic effects of ANF at all dose levels and the reduction in ANF effects was much less than that observed in pregnant rats.

In addition, the cGMP response to increasing doses of ANF were similar in virgin and progesterone-treated rats (Fig. 3.16) suggesting that progesterone does not affect GC-linked ANF receptors; however, this study does not preclude the possibility that progesterone might exert its influence at a site beyond cGMP formation.

Thus, it seems difficult to definitively establish the role that progesterone might play in attenuating the renal effects of ANF given the above described inconsistencies; however, these inconsistencies may be due to the possibility that it is difficult to experimentally reproduce the complex hormonal changes and their temporal profiles during pregnancy. Certainly, one cannot claim our technique of once a day injections of progesterone or estrogen in intact or OVX-animals can reproduce the relative balance between estrogen and progesterone during pregnancy. In addition, any independent effect of progesterone (95, 96, 99, 365) and estrogen (366) on renal sodium and water handling may have further complicated the results of this study. It is noteworthy to mention that some workers (340, 341), have suggested that the high circulating levels of aldosterone during pregnancy are secondary to the high progesterone levels (340). In one study (340), plasma concentration of the two hormones correlated significantly and the administration of progesterone to nonpregnant volunteers or to women with dead fetuses but intact placenta increased plasma aldosterone levels; however, the increments in plasma aldosterone were less than that observed in pregnancy.

In conclusion, this study demonstrates that rat pregnancy is associated with an attenuation of the diuretic and natriuretic effects of ANF; this decrease in the renal effects of ANF is conducive to the fluid expansion that accompanies gestation. Our findings also reveal that the refractoriness of the gravid rat to the renal effects of ANF are neither due to an increase in the *in vivo* degradation of ANF nor to increased renal cortical endopeptidase activity but due to a decrease in the ANF GC-A receptors and receptor mRNA and the effects of ANF on cGMP production. The downregulation of renal ANF receptors and hence in its effects might be a physiological adjustment to allow fluid/electrolyte expansion during pregnancy.

Although ANF exerts profound effects on the kidney, adrenal glands and cardiovascular system (119), so far no pathologic state has been identified to be due to excess or deficiency of ANF. The modulation of the renal effects of ANF as well as the downregulation of its receptors during pregnancy in the face of an expanding plasma volume, imply that the fluid and electrolyte homeostatic mechanisms within the body regulate ANF according to the physiological needs. Perhaps the dynamic adjustments between the physiologically required plasma volume and the functions of ANF are important reasons why a pathologic state does not progress because of dysfunction of the ANF system. Moreover, a lack of association of ANF with any disease in way undermines the role of ANF as a modulator of fluid-electrolyte homeostasis.

CHAPTER FIVE: REFERENECES

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