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ATRIAL NATRIURETIC PEPTIDE

Adriaan C.I.T.L. Tan

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Geneeskunde en Tandheelkunde
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Aan mijn vader
en mijn moeder

Contents

Part I

INTRODUCTION

Chapter 1.1
General introduction to the topic

Atrial natriuretic peptide, a hormone secreted by the heart, has come to be considered a major contributor to the hormonal control of the cardiovascular system. Although the endocrine function of the heart was demonstrated only in 1981, an association between the cardiac atria and renal function had been suspected long before.

As early as 1935 Peters speculated on the existence of a volume controlling system which could 'sense the fullness of the blood stream' (1). In 1956 Henry, Gauer and Reeves (2) suggested that the heart could detect changes in the central blood volume. Inflation of a balloon in the left atrium of dogs was shown to increase diuresis, natriuresis and urine flow rate (3,4). Initially these findings were attributed to a reactive decrease in the secretion of vasopressin or a response of the nervous apparatus (5,6). However, the diuresis and natriuresis induced by volume expansion were found not to be inhibited by cardiac denervation, by vagal sectioning, by destruction of the pituitary or by antidiuretic hormone infusion (7-9). These observations did not preclude the possibility of a blood-borne diuretic and natriuretic factor that could explain the observed phenomena.

In 1961 deWardener and colleagues performed a series of experiments that strongly pointed to the presence of a circulating natriuretic factor. In these experiments an isolated dog kidney was perfused with blood circulating through a second dog in which saline volume expansion was induced. This resulted in a marked increase in the urine flow rate from the isolated dog kidney (10). This phenomenon suggested to them the existence of an unidentified 'third factor' affecting sodium excretion, as collateral experiments showed that the natriuresis resulting from sodium load occurred independently of changes in glomerular filtration rate (first factor) and aldosterone (second factor) (10). This 'third factor' assumed to be a circulating humoral mediator, came to be known as the natriuretic hormone. In contrast to atrial natriuretic peptide this substance, of a low molecular weight <1000 Da (11), considerably lower than that of atrial

natriuretic peptide (3081 Da), inhibits Na^+/K^+-ATPase activity (12) and possesses ouabain-like immunoreactivity (13). Its exact chemical structure has not yet been elucidated.

Concurrently with the experiments which demonstrated that both volume expansion and increase in atrial pressure induce diuresis, possibly through the medium of a circulating agent, an entirely different line of research was followed which led to the discovery of the atrial natriuretic peptide. In 1951 Kisch and colleagues using an electron microscope detected granules in atrial myocytes which were absent in ventricular myocytes (14). Jamieson and Palade noticed the resemblance of these granules to secretory granules found in endocrine organs (15). In 1979 deBold and colleagues found that the amount of these specific granules in the atria was negatively correlated with the volume of extracellular fluid (16). The number of these granules increased by water deprivation and decreased by saline loading or administration of deoxycorticosterone. In 1981 these investigators performed a crucial experiment: they injected atrial extracts into rats and observed massive diuresis and natriuresis. In contradistinction, ventricular extracts did not produce these effects (17). DeBold and colleagues concluded that the heart secretes a hormone which is stored in specific atrial granules and is involved in the volume regulation of the cardiovascular system. In view of its site of production, its effects and chemical structure this hormone was called 'atrial natriuretic peptide (ANP)'.

In the past few years extensive biomedical research has elucidated the structure and function of this peptide. The next chapter gives a brief review of the current knowledge regarding this new peptide hormone.

Chapter 1.2

Survey of the literature and scope of the present study

Isolation and characterization of ANP

Soon after the landmark experiment by deBold *et al.* (17) several investigators isolated various related polypeptides from the atrium (18,19). These polypeptides possess natriuretic, diuretic and vasorelaxant properties. All these peptides were found to derive from a common precursor peptide containing 126 amino acids (20) (figure I−1). As different investigators reported isolation of these peptides, different names were initially given to them, which led to confusion and complicated scientific communication. Therefore a nomenclature committee, established jointly by the International Society of Hypertension, the American Heart Association and the World Health Organization, has recommended that the term 'Atrial Natriuretic Factor (or Peptide)' be adopted and that the prefix 'h' for human or 'r' for rat be used for species designation (21). Furthermore the specific amino acid composition of each peptide should be designated with reference to the common precursor molecule, with peptide sequence starting at the N-terminus. Thus the precursor form of ANP is (1-126)ANP (also known as γ−ANP). In blood, the major circulating form of ANP is (99-126)ANP (also known as α−ANP), made up by the final 28 amino acids on the C-terminal end of (1-126)ANP (22,23).

Evidence is accumulating that besides (99-126)hANP, other peptides deriving from (1-126)hANP circulate in human blood. The presence of the precursor peptide itself as well as cardiodilatin (corresponding to (1-98)hANP) and ß-hANP (an antiparallel dimer of (99-126)hANP) have been reported (24,25). Also, shorter fragments of (99-126)hANP have been found in human blood (26,27) (figure I−2). As the incidence of these peptides seems to differ in various pathological conditions (28), the possibility cannot be excluded that altered atrial processing and secretion of abnormal forms may be involved in certain disturbances of sodium and fluid balance.

The major circulating form, (99-126)hANP possesses a disulfide bridge between the two cysteine residues, which is essential for its biological activity (29) (figure I−3).

Successive deletion of the five C-terminal residues gradually decreases the biolo-

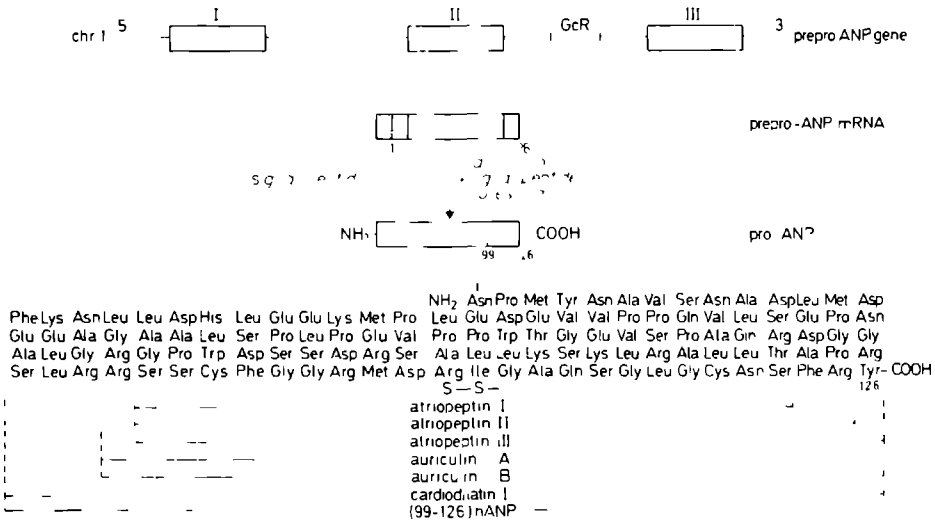

Figure I-1. The gene for ANP coding for (1-126)hANP.

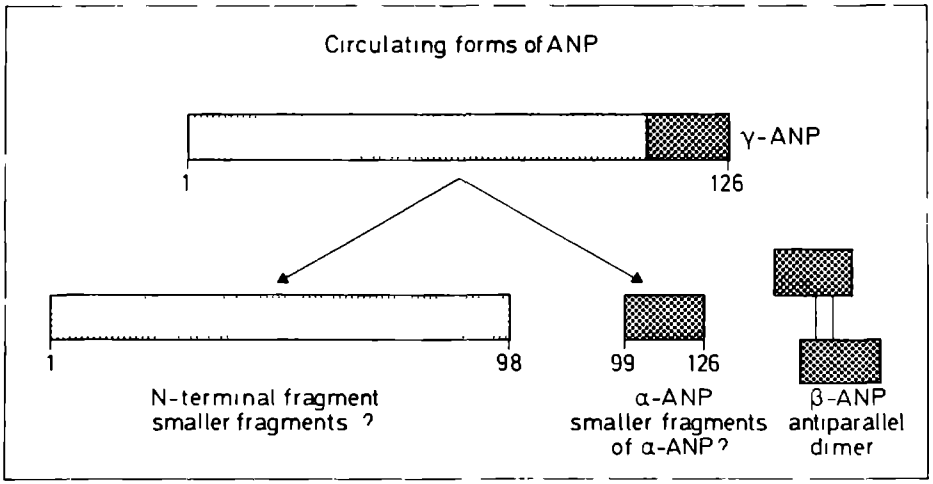

Figure I-2. Within the circulatory system various fragments derived from (1-126)hANP (or γ-hANP) are found.

gical activity of ANP, while deletion or prolongation of the N-terminal residues seems to have little effect (30,31). Human (99-126)ANP differs from rat

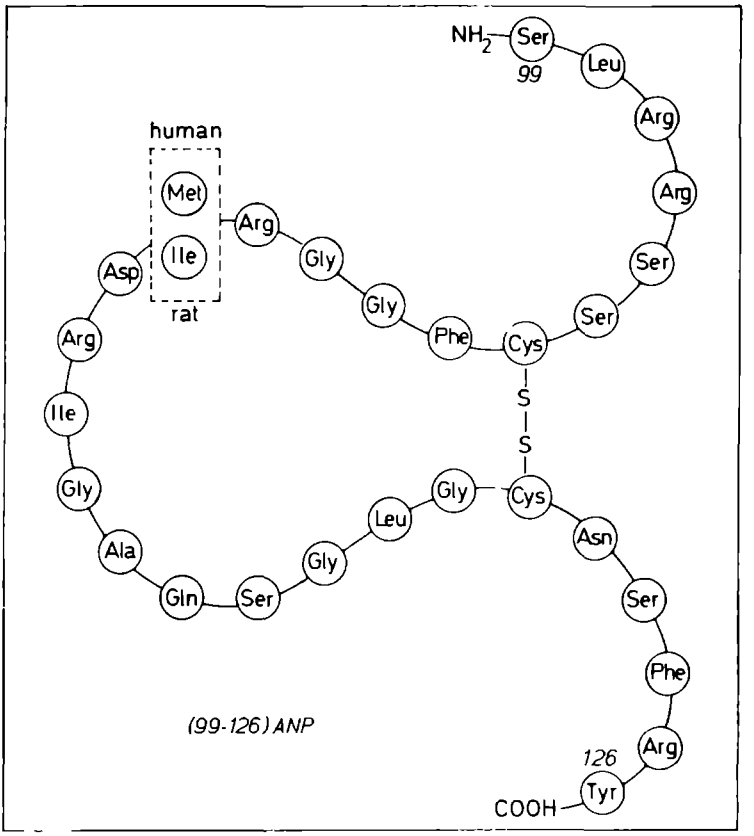

Figure I-3. Structure of the major circulating component in blood: (99-126)ANP.

(99-126)ANP by one amino acid. Where in rat position 110 is occupied by isoleucine it is occupied by methionine in man.

The gene for ANP consists of three translated sequences (exons) and two intervening sequences (introns) not coding for the precursor peptide (figure I−1). It is of interest to note that the second intron contains a sequence for a putative glucocorticoid receptor-regulatory element (GcR) (32,33). This suggests that the synthesis of ANP may be partly regulated by glucocorticoids. Accordingly, when fludrocortisone or prednisolone was administered to healthy persons, treatment with the mineralocorticoid fludrocortisone induced volume expansion (explaining the rise in ANP levels) with weight gain, whereas treatment with the glucocorticoid prednisolone did not induce such effects. Both treatments however induced similar increases in ANP levels (34). When dexamethasone was added to

atrial myocytes *in vitro*, the immunoreactive ANP level in the myocytes, the mRNA level and the amount of ANP released from atrial myocytes all increased (35).

The gene for ANP encodes for a 151 amino acids pre-pro peptide. After cleavage of the 25 amino acids hydrophobic signal-peptide, the (1-126)ANP pro-peptide arises. The main storage form of ANP as found in granules in atrial myocytes is (1-126)ANP while the major circulatory form is (99-126)ANP (36). The cleavage of the Arg_{98} - Ser_{99} bond to produce the bioactive (99-126)ANP form probably occurs before the release of ANP into the bloodstream (37,38). The identification in bovine and rat atria of a protease specific for the putative cleavage site indicates that the atrial tissue contains the enzyme required for the proteolytic activation of pro-ANP (39,40).

Under normal physiological condititions immunoreactive ANP is present in both atria, but predominantly in the right one (41) which also contains a greater number of secretory granules (42). Atrial appendectomy markedly diminishes the natriuretic response to volume load (43,44).

Chronic volume overload as occurs in congestive heart failure, may lead to depletion of atrial ANP and 'recruitment' of ventricular myocytes. The ratio of atrial versus ventricular ANP, which is normally about 1000:1, can under these conditions decrease to 10:1. The amount of mRNA in both atrium and ventricle increases and secretory granules appear in the ventricles (45-48). In the normal state no storage granules in ventricular myocytes are found, which implies that the ventricular myocytes secrete ANP via a constitutive rather than a regulatory secretory pathway like the atrial myocytes (48,49).

Release of ANP

The first indications for the existence of a blood-borne natriuretic substance came from the observations made by deWardener and colleagues that blood from a volume expanded dog could induce natriuresis (10). As mentioned above, these investigators attributed this phenomenon to a putative substance, designated 'natriuretic hormone'. It is however more likely that this phenomenon is mediated at least in part by the atrial natriuretic peptide. Indeed, after the experiment by deBold *et al.* in 1981 (17) it was shown, in human and animal studies, that volume expansion is associated with a marked rise in ANP levels and concomitant diuresis and natriuresis (50,51). The diuretic/natriuretic response to volume expansion in rats can be blocked by administering monoclonal ANP-

antibodies, which by binding to ANP neutralize its action (52-54). The hypothesis that the blood volume determines the release of ANP is supported by the observed positive correlation of ANP concentrations with the right atrial pressure (55,56). Although in general atrial pressure is a measure for atrial stretch, it is the stretch rather than the pressure that seems to be the main stimulus for release of ANP (57,58). The pivotal role played by atrial stretch implies that it is the central (or so-called 'effective') blood volume rather than intravascular volume that is the main determinant of ANP release. Manoeuvres causing a redistribution of intravascular fluid volume such as head down tilt, head out water immersion or change from the upright to the supine position increase ANP concentrations (59,60). The increase in ANP concentrations observed during infusion of pressor agents like angiotensin II or phenylephrine probably results from increased right atrial pressure rather than from a direct effect of angiotensin II or phenylephrine on ANP release (61). The amount of sodium in the diet influences ANP release. High sodium intake for 3 to 5 days can increase ANP concentrations two to fourfold. A close positive correlation was observed between body weight gain, fractional sodium excretion and plasma ANP concentration, which shows that ANP plays a role in regulating the body fluid balance (62,63). ANP increases during tachycardia or rapid atrial pacing in proportion to heart rate (64), which in all likelihood is due to atrial stretching. Interestingly, repeating the stimulus seems to enhance ANP release (65).

As ANP levels reflect intracardiac pressures, high levels have been observed in pathological conditions associated with volume overload such as congestive heart failure, chronic renal failure, primary aldosteronism or the syndrome of inappropriate secretion of antidiuretic hormone (ADH). With normalization of extracellular fluid volume (e.g. by drug treatment or haemodialysis) ANP concentrations fall (55,66-70). The somewhat blunted response to infusion of ANP in congestive heart failure may be due to diminished responsiveness of target-organs to ANP, to intrinsic renal dysfunction or to activation of sodium-retaining systems offsetting the effects of ANP (71).

After the discovery of ANP it was speculated that impaired secretion of or impaired response to this hormone could be involved in the pathogenesis of hypertension. However, both in experimental and human hypertension an elevation in ANP concentrations was found rather than a reduction. While strongly elevated ANP concentrations were measured in severe hypertension accompanied by left ventricle hypertrophy, positively correlating with blood pressure, normal or only slightly elevated concentrations were observed in mild essential hypertension (72,73). Infusion of synthetic ANP in humans with essential

hypertension has an enhanced blood pressure lowering effect, attended with enhanced diuresis and natriuresis (74). This does not suggest reduced organ responsiveness to ANP in essential hypertension. Although the above mentioned data do not support the idea of a dysfunction of the ANP system in hypertension, it is interesting to note that sons of hypertensive parents, as compared to sons of normotensive parents, exhibit a diminished ANP response to a 7 day high sodium diet (75). Hypertensive patients, as compared to normal persons, exhibit an enhanced natriuresis and an enhanced ANP response to saline infusion (76). Exercise increases heart rate, blood pressure and ANP release more in hypertensives than in normals, while the rise in ANP was found to correlate with the degree of left ventricular dysfunction (77,78).

Effects of ANP

The potent effects of ANP on the kidney led to intensive research into the mechanisms involved. Infusion of the synthetic peptide into animals or humans causes large increases in urinary sodium and chloride excretion, together with moderate increases in calcium, magnesium and phosphate excretion with little change in potassium excretion and free water clearance (79-81). The distribution of ANP receptor sites in the kidney suggests that ANP acts mainly on the glomeruli, collecting ducts and the renal vasculature (82,83). The renal effects of ANP seem to be largely due to its renal haemodynamic and vascular activities, in particular to an increase in glomerular filtration rate and filtration fraction, a medullary washout and a redistribution of renal blood flow away from the salt retaining nephrons. ANP can increase both single nephron and whole kidney glomerular filtration rates, probably by constriction of efferent and dilatation of afferent glomerular arterioles together with increased glomerular capillary permeability (84-87). A direct effect of ANP on the renal tubulus cannot be ruled out (80,88,89).

Infusion of ANP causes a dose-dependent fall in the arterial blood pressure in man as well as in animals (81,90,91). This may be caused by a direct vasodilating effect of ANP since isolated vessels or vascular preparations partially constricted by the application of pressor agents relax when ANP is added (92,93). Infusion of synthetic ANP into humans, however, does not decrease but increases peripheral resistance. This is probably due to reflex activation of the sympathetic nervous system. The blood pressure lowering effect of ANP may thus be due to a decrease in cardiac output (94,95). Such decrease cannot be explained by a direct negative effect of ANP on the heart, as ANP does not affect the force of

contraction nor the heart rate in isolated cardiac atria (96). Furthermore ANP increases coronary blood flow, and during infusion of ANP right atrial pressure decreases rather than increases as would be expected in the case of a negative inotropic effect (97,98). The reduction in cardiac output can be ascribed to a decrease in venous return following ANP infusion (97). Diuresis and contraction of the intravascular blood volume may play an additional role. ANP causes a reduction in the circulating blood volume due to a shift of intravascular fluid to the interstitial space, which shift presumably results from increased capillary hydraulic conductivity and postcapillary resistance (99,100). This reduction is reflected in a rise in haematocrit, a phenomenon which cannot be ascribed to increased diuresis since it is also found in anephric rats (101).

Yet, some investigators observed an increase in cardiac output during infusion of ANP (102). The change in cardiac output may depend on the degree of afterload reduction (which increases cardiac output) and preload reduction (which decreases cardiac output). The contradictory observations regarding the cardiovascular effects of ANP may be attributable to species-related differences, to the level of consciousness of the experimental subject, and to the molecular form of ANP infused. Furthermore the dose of ANP, the mode of administration (e.g bolus or constant intravenous infusion, or both) and the duration of infusion varied widely with different experimental protocols. Besides, the medium in which ANP is dissolved and the study design (whether or not placebo-controlled) are to be taken into consideration.

ANP can be considered to be a functional antagonist of the renin-angiotensin-aldosterone system. It inhibits the rise in PRA after head-up tilt (103). Low dose ANP infusion decreases PRA secretion (80,90,91). High dose ANP may stimulate PRA, presumably by reflex activation of the sympathetic system. In cultured juxtaglomerular cells renin release was inhibited by ANP (104). Also the increased delivery of sodium to the distal tubulus may decrease renin secretory rate from the macula densa cells. Aldosterone synthesis is directly inhibited by ANP at a step preceding the mitochondrial metabolism of cholesterol (105). Plasma cortisol was reported to decrease after administration of ANP (106) but not consistently (80,90). Haemorrhage- or dehydration-induced release of ADH was inhibited by ANP in the rat (107). Also, the rise in ADH concentrations after head-up tilt (103) and the osmolality-induced ADH release (108) were inhibited by ANP. It was shown that ANP reduces the rate of ADH release *in vitro* (109). Several investigators did not detect any change in ADH concentrations *in vivo* (79,81), while others noted a slight decrease during infusion of ANP or, after it, a rebound increase implying suppression of ADH during in-

fusion (110).

ANP may influence the sympathetic nervous system. Low doses of ANP in humans do not affect the release of catecholamines (91,95). In dog, low doses of ANP lower blood pressure slightly and decrease the release of catecholamines, whereas higher doses of ANP tend to have a reverse effect. This implies that low infusion rates of ANP may inhibit the release of epinephrine from the adrenal medulla and also the baroreceptor reflexes that normally increase norepinephrine release. At larger doses of ANP the more pronounced hypotensive effects elicit a more powerful reflex response of the sympathetic nervous system, thereby increasing norepinephrine release (94,111).

Figure I–4 illustrates the function of ANP in the regulation of the extracellular fluid (ECF) volume. Distension of atrial myocytes by increasing ECF volume stimulates cardiac ANP secretion. The various effects of ANP on its target-organs (kidney, adrenal and cardiovascular system) lead to a decrease in ECF volume which by diminishing atrial distension decreases ANP secretion.

Receptor and second messenger of ANP

Autoradiographic and receptor binding studies have revealed an abundance of binding sites for ANP in the body. ANP binding sites are localized in the kidney, adrenal gland, small intestine, colon, certain brain areas and blood vessels (112-114). This wide distribution of binding sites is not surprising in view of the role of ANP in controlling salt and water balance and blood pressure.

The ANP receptor population is functionally heterogeneous. Two types of receptors exist: one with and one without guanylate cyclase activity. Both guanylate cyclase-containing and guanylate cyclase-free receptors possess similar molecular weights of approximately 130.000 daltons under nonreducing conditions. Under reducing conditions the cyclase-free receptor consists of subunits of about 65.000 daltons (115,116). The difference between the two types of receptors is also expressed in their binding capacity to ANP and its analogues: while the guanylate cyclase-containing receptor (called B-receptor) binds ANP molecules containing more than 24 amino acid residues, the guanylate cyclase-free receptor (C-receptor) is capable of binding truncated ANP analogues with 21 or 17 (ring) amino acid residues (117). The development of specific ANP analogues for the C-receptor made it possible to clarify their function. Although these analogues bind to more than 95 % of (99-126)ANP binding sites in the

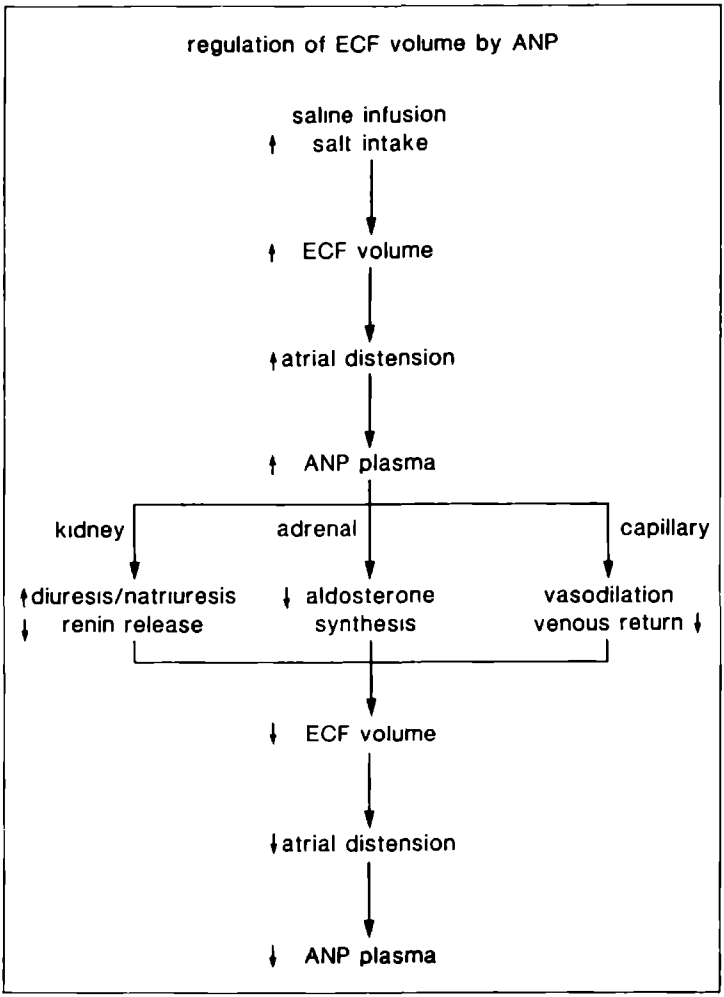

Figure I-4. Schematic drawing of the function of ANP in the regulation of extracellular fluid (ECF) volume.

kidney, no cyclic guanosine monophosphate (cGMP) was produced (118). In the isolated rat kidney these analogues exhibit no effects on renal vasculature, glomerular filtration rate, filtration fraction or excretion of fluid and electrolytes. On the other hand, when these analogues were infused into intact animals, a marked diuresis and natriuresis occurred. Blood pressure decreased and glomerular filtration fraction increased. Concomitantly, plasma levels of (99-126)ANP increased. These findings indicate that the natriuretic and blood

pressure lowering effects of the analogues should not be ascribed to their intrinsic ANP nature but to their ability to increase plasma levels of endogenous (99-126)ANP (118). As these analogues compete with endogenous (99-126)ANP for binding to C-ANP receptors, it is likely that the C-ANP receptor modulates the metabolic clearance of ANP (119).

After binding of ANP to its receptor the membrane-bound enzyme particulate guanylate cyclase is activated and cGMP is produced (120,121). Cyclic GMP, acting as second messenger for ANP then provokes the biological effects of ANP through the cGMP-dependent protein kinase (122,123). The receptor and the enzyme particulate guanylate cyclase are tightly coupled and probably represent the same entity (124,125). When the actions of ANP are blocked by antibodies directed against ANP, no cGMP production is observed (126,127). While addition of a permeable analogue of cGMP *in vitro* and *in vivo* mimicks the effects of ANP (128-130), prevention of cGMP production abolishes the effects of ANP (131,132). Cyclic GMP values can be measured in plasma as well as in urine. Measurement of the cGMP response to ANP may be useful to investigate the responsiveness of the target organs to ANP (133,134).

ANP-peptides in the brain and the kidney

ANP-like immunoreactive material has been found in various parts of the brain and the peripheral nervous system. The highest concentration of ANP, though 3000-fold lower than in the atria, is found in the hypothalamus. Local production of ANP in the brain is suggested by the existence of ANP mRNA transcripts in rat brain, especially in the hypothalamus. *In vitro*, the rat hypothalamus can release ANP-like material (135-139). These observations point to a neurotransmitter or neuromodulator role of ANP in the central nervous sytem. While in blood the predominant form of ANP is (99-126)ANP, in rat brain the predominant forms are (102-126)ANP and (103-126)ANP, which implies different processing and maturation of the ANP prohormone in the brain (139,140). Specific receptors for ANP have been demonstrated in brain tissue. Brain microvessels constituting the blood-brain barrier also possess specific ANP receptor binding sites. As ANP is able to alter the rate of production of cerebrospinal fluid, it may participate in the constant exchange of fluid between blood and brain (141,142).

Injections of ANP into the lateral ventricle of rat brain induces multiple effects on the regulation of water and salt balance. ANP increases urinary volume, de-

creases salt and water intake and inhibits ADH secretion and the pressor response to angiotensin II in rats. Intracerebro-ventricular injection of antiserum to ANP enhances angiotensin II-induced water intake (143-149). Thus ANP in the brain acts synergistically with its known effects as a cardiovascular hormone.

Recently, a novel 26 amino acids peptide called 'Brain Natriuretic Peptide' (BNP, figure I–5) was identified in porcine brain. Its structure strongly resembles that of ANP but it definitely is neither a precursor nor a degradation product of it (150-152). Correspondingly, BNP does not crossreact with ANP in a radioimmunoassay (153). Although the localization of BNP in rat brain seems to differ from the localization of ANP (154), the two peptides share the same receptor, as shown by receptor displacement studies (151,155). BNP, like ANP, acts on particulate guanylate cyclase and increases cGMP (151,156). The effects of BNP are similar to those of ANP: inhibition of adrenal steroidogenesis (156), diuretic, natriuretic, blood pressure lowering and vasorelaxant activities (150), antidipsinogenic action (157) and inhibition of ADH secretion (158). Interestingly, BNP seems to be present in the atrium as a prohormone and to be secreted into the circulation as a smaller molecule, resembling the ANP hormone. The amount of immunoreactive BNP was found to be approximately 1-5 % of the amount of immunoreactive ANP in porcine heart and plasma (153).

The kidney, besides being a major target-organ for ANP, may also be influenced by paracrine hormones. Urodilatin, a 32 amino acid hormone corresponding to (95-126)ANP, was identified in the collecting ducts of the rat kidney (figure I–5). Messenger RNA for pro-ANP was found in the collecting duct, which suggests local production and processing of this hormone. Preliminary reports indicate that urodilatin administered to rats induces effects similar to those of ANP (159).

It is becoming increasingly clear that the atrial natriuretic peptide hormone system can be regarded as antagonistic to the renin-angiotensin aldosterone system. The circulating atrial natriuretic peptide in blood, the paracrine hormone urodilatin and the brain natriuretic peptide exert functions opposite to those of the latter hormone system. They increase diuresis and natriuresis, lower blood pressure, induce vasodilation, and suppress renin, aldosterone and vasopressin secretion. Furthermore the existence of a circulating hormone, a paracrine hormone and a putative neurotransmitter point to the integrated function of this hormonal system in the control of body fluid homeostasis.

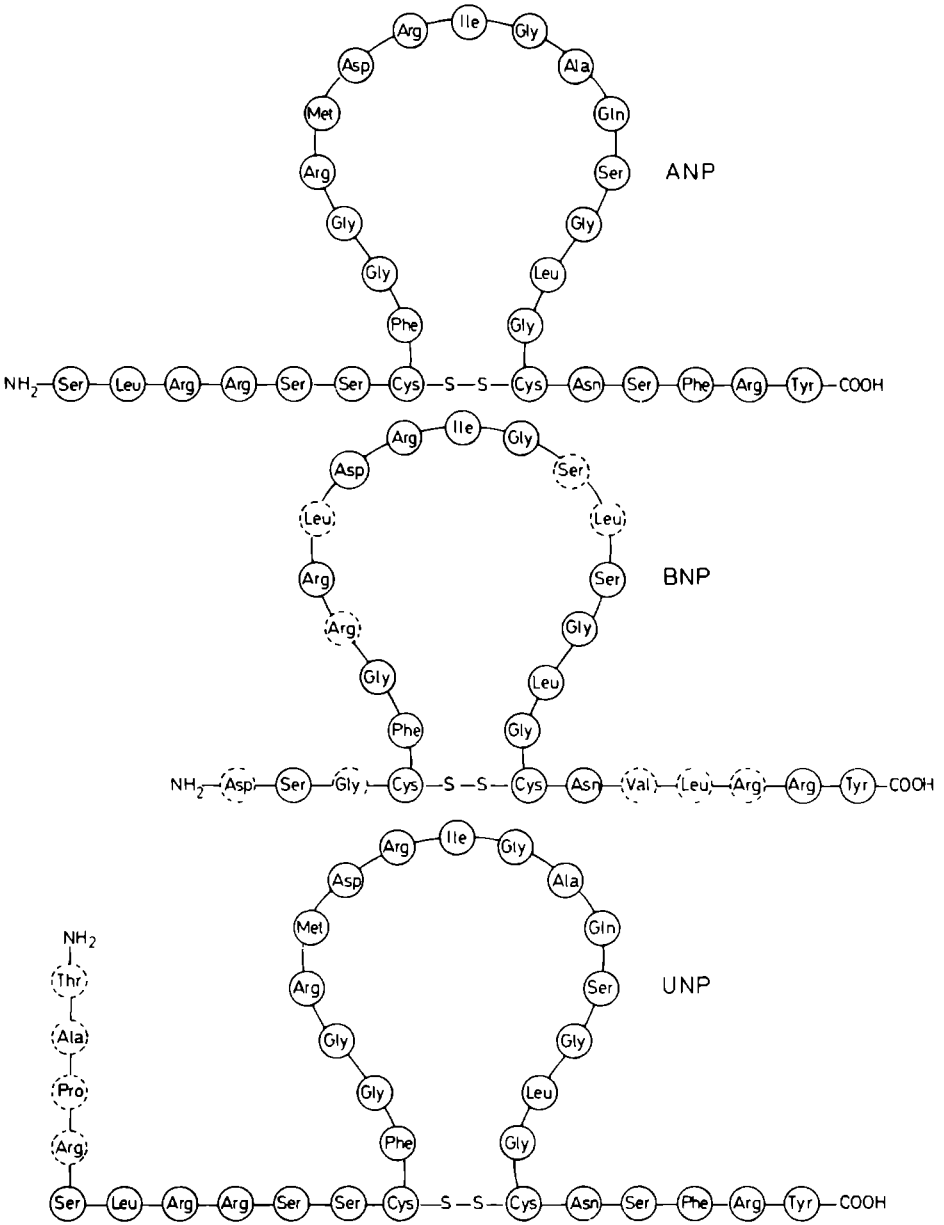

Figure I-5. The marked similarity between atrial natriuretic peptide (ANP), porcine brain natriuretic peptide (BNP) and urodilatin (UNP) point to the integrated function of this hormonal system in the control of body fluid homeostasis. Note that urodilatin corresponds to (95-126)hANP.

Scope of the thesis

The investigations described in the following chapters were performed with a view

- to develop and validate a method for the determination of ANP concentrations in human blood (Part II)
- to study the influence of various physiological and sampling conditions affecting the measurement and interpretation of ANP concentrations (part III)
- to explore the physiological relevance of the higher ANP values found in elderly subjects as compared to those measured in young subjects, including a number of observations on the second messenger of the ANP hormonal system, cyclic guanosine monophosphate (part IV)
- to study the involvement of the atrial natriuretic peptide system in pulmonary embolism and myocardial infarction (part V)

In this thesis the abbreviation ANP is used to designate immunoreactive atrial natriuretic peptide in humans.

Chapter 1.3
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Part II

MEASUREMENT OF ANP CONCENTRATIONS

In part I of this thesis a brief analysis of the literature regarding the function of the atrial natriuretic peptide is given. The atrial natriuretic peptide (ANP) hormonal system can be regarded as antagonistic to the renin-angiotensin-aldosterone system, its main function being to protect the body from cardiovascular volume overload. As ANP values reflect the volume of the intravascular compartment, measurement of ANP concentrations can be of value to determine the role of ANP in the endocrine control of the cardiovascular system and in various pathophysiological states in man. In this part of the thesis two methods for measurement of ANP concentrations are described and compared. Furthermore, using high pressure liquid chromatography analysis an approach to determine the molecular forms of immunoreactive ANP in human plasma is described. As cyclic guanosine monophosphate (cGMP) functions as the second messenger for ANP, the cGMP concentration in plasma reflects the biological effectiveness of ANP. Therefore a method for measurement of cGMP concentrations in human plasma was developed.

Chapter 2.1

Extraction method

Introduction

Soon after the sequence analysis of ANP and the first radioimmunoassay of ANP in plasma (1), synthetic (99-126)hANP and antibodies directed against ANP became commercially available. This enabled various investigators to establish a method for measurement of ANP in human blood.

These methods vary widely; both direct assays (2-5) and assays using an extraction step have been described. Prior extraction of plasma can be achieved using Vycor-glass (6), Sepharose-coupled antiserum (7-9) or silica cartridges (10-15). In order to develop a method for measurement of ANP concentrations in man we immunized sheep to obtain a specific antibody directed against (99-126)hANP. As the obtained antibodies were specific for the non-oxidized form of (99-126)hANP, a suitable non-oxidized tracer was obtained after comparing two different iodination procedures. Using these antibodies and tracer a sensitive and reliable method for measurement of ANP based on prior extraction of the plasma on Seppak C-18 columns was developed.

Materials and methods

Materials

Synthetic (99–126)hANP (for constructing standard curve and iodination), (103-126)hANP, (105-126)hANP, (111-126)hANP, (116-126)hANP, and (99-109)hANP were purchased from Peninsula Laboratories Inc, England; rat (99-126)ANP was a gift from Dr. B. Penke, University of Szeged, Hungary; alpha-MSH, (Arg)-vasopressin and ACTH were obtained from INCSTAR, Stillwater, MN, USA; $Na^{125}I$ was from Amersham Int, Amersham, UK; aprotinin (Trasylol) was from Bayer, Leverkusen, FRG; sheep IgG, Bovine Serum Albumin (BSA Cohn fraction V) and Triton X–100 were from Sigma Chemicals Corp, St. Louis, MO, USA; PEG–6000, EDTA, H_3BO_3 and NaOH were obtained from Merck, Darmstadt, FRG; Protag-125, ethanol, acetonitrile (HPLC-grade), acetic acid, methanol (HPLC-grade), distilled water (HPLC-grade) and trifluor acetic acid (TFA, HPLC-grade) were obtained from Baker Fine Chemicals, USA.

Complete Freund's adjuvant was purchased from Hoechst, The Netherlands. All reagents were of analytical grade, except where mentioned otherwise. To obtain a tracer with preferable binding characteristics, as compared to a chloramine-T product, a Protag iodination was performed.

Blood collection

Blood samples (3 ml) were collected by venepuncture into EDTA vacuum tubes, kept on ice, centrifuged as soon as possible (1500 g, 4°C, 10 minutes), and stored in polystyrene tubes containing 200 KIU of aprotinin (Trasylol, Bayer AG, FRG)/ml plasma at −80°C until used for extraction.

Extraction

Seppak C-18 cartridges (Waters Associates, Milford, MA, USA) were pre-treated by subsequently washing with 5 ml acetic acid(96 %)-ethanol(86 %) (4:96 v/v), 5 ml methanol, 5 ml distilled water and 5 ml acetic acid(96 %)-distilled water (4:96 v/v). One ml plasma was acidified with 3 ml acetic acid (96 %)-distilled water (4:96 v/v) in a polystyrene tube and applied to the cartridge. After washing twice with 3 ml distilled water, the ANP was eluted in polystyrene tubes with 3 ml acetic acid(96 %)-ethanol(86 %) (4:96 v/v) (modified from Lang *et al.* (16)). The eluant was evaporated under a stream of nitrogen for 1 hour at 37°C. The walls of the tubes were rinsed with 1 ml ethanol (100 %) and evaporated to dryness. The residue was dissolved in 0.50 ml RIA-buffer (0.2 mol/l sodium borate (pH 8.4), 10 mmol/l of EDTA, 0.1 % Triton X-100 detergent and 2 g/l BSA) and radioimmunoassayed. Extraction efficiency was assessed by recovery experiments in which different amounts of (99-126)hANP were added to four different plasmas. The effect of evaporation was assessed by evaporating six samples of pure standard in either elution solvent or standard added to plasma or plasma extracts. Comparisons were made using the Wilcoxon two-sample test.

Antibodies against (99-126)hANP

The immunogene used for production of antibodies against (99-126)hANP was kindly provided by INCSTAR, Stillwater, USA. It was prepared by conjugation of synthetic (99-126)hANP to keyhole limpet haemocyanin using carbodiimide as the coupling agent (17). Two mg of the conjugate was dissolved in 1 ml saline and emulsified with an equal volume of complete Freund's adjuvant. Two Texel white sheep (S−31 and S−32) were immunized subcutaneously in two places on their back. Every 2 weeks, booster injections were given with the same composition as the first immunization. At the same time, blood was drawn for determination of

the antiserum-titre and for study of the cross-reactivities.

Antibodies against sheep-IgG

Horses were immunized subcutaneously with 2 mg sheep-IgG, which was dissolved in 1 ml saline and emulsified with 1 ml complete Freund's adjuvant. Every 2 weeks booster injections of the same composition were given and blood was drawn for titre measurements.

Preparation of [^{125}I]-monoiodotyrosyl-(99-126)hANP

Two methods of preparation of [^{125}I]-(99-126)hANP were compared; the Protag-125 iodination method according to Guenther *et al.* (18) and the chloramine–T method, modified from Greenwood *et al.* (19). Synthetic (99-126)hANP was iodinated with Na^{125}I using either Protag-125 or Chloramine–T. Subsequently the iodination mixture was loaded onto a 3 ml silica C–18 column (Baker Fine Chemicals, USA), which was previously prewashed using 4 ml methanol, 4 ml distilled water and 4 ml phosphate buffer (67 mmol/l, pH 7.4). After washing with 3 ml solvent A (methanol-distilled water-TFA 20:80:0.1 v/v/v), elution was carried out with 1 ml solvent B (methanol-distilled water-TFA 80:20:0.1 v/v/v). The eluate was purified by high pressure liquid chromatography (HPLC) using a micro-Bondapack column (20 cm), with a flow rate of 2 ml/min. The initial solvent composition was acetonitrile-distilled water-TFA (20:80:0.1 v/v/v). A linear gradient from 20 to 50 % acetonitrile was performed to elute the different iodinated forms of ANP. [^{125}I]Monoiodotyrosyl-(99-126)hANP eluted at 30 % acetonitrile.

Radioimmunoassay

One hundred microlitres of plasma-extract or standard in RIA-buffer was mixed with 200 µl of sheep anti-ANP serum (S–32, final dilution in RIA-buffer 1:2.5 x 10^6) at 4°C in polystyrene tubes. The mixture was allowed to incubate for 18 hours at 4°C. Two hundred microlitres of tracer solution (9500 dpm) was added, and incubated for a further 24 hours at 4°C. Separation of bound and free hormone was achieved by addition of 1.0 ml horse anti-sheep precipitating complex (1 µl normal sheep serum, 15 µl horse anti-sheep-IgG serum and 30 mg PEG-6000 per ml sodium borate buffer (20 mmol/l, pH 8.4, 2 g/l BSA)) at room temperature for 30 minutes. Finally the suspension was centrifuged for 30 minutes at 1000 g at room temperature. The tubes were decanted and the radioactivity of the pellet was counted in a gamma counter. Parallelism was assessed by assaying 0.25, 0.50 (both made up to 1.0 ml with RIA-buffer) and 1.0 ml of different plasma samples.

Results

Antibodies against (99-126)hANP

Both sheep produced antibodies with very high titres (up to 1:2.5 x 10^6 final dilution). After two months, no further increase in titre was found. In the final assay procedure, the antibody designated S−32 was used, which was collected after 5 months of immunization from sheep S−32. The other antibody, S−31, was used in the direct assay for ANP (see chapter 2.2). In table II−1 the cross-reactivities of the S−31 antibody (direct method), the S−32 antibody (extraction method) and a commercially available antibody from Peninsula are shown. The antibodies S−31 and S−32 recognize only the ANP peptides possessing an intact disulfide bridge. Complete cross-reactivity was found with ß−hANP. No cross-

Table II-1. Cross-reactivities of the S-31, the S-32 and the Peninsula antibodies (percentages vs. (99-126)hANP).

	S-31	S-32	Peninsula
(99-126)hANP	100	100	100
(99-126)rANP	1.5	2.2	100
(103-126)hANP	104	109	90
(105-126)hANP	117	127	95
(101-126)hANP	107	127	125
(99-109)hANP	<0.2	<0.2	<0.2
(111-126)hANP	<0.2	<0.2	65
(116-126)hANP	<0.2	<0.2	55
ß-hANP	-	100	-
(1-30)hANP	-	<0.2	-
(31-67)hANP	-	<0.2	-
α-MSH	<0.2	<0.2	<0.2
ACTH(1-39)	<0.2	<0.2	<0.2
Arg^8-Vasopres.	<0.2	<0.2	<0.2

reactivity was observed with different parts of (1-126)hANP not containing the (99-126)hANP fragment. In contrast to the Peninsula antibody, both the S−31 and the S−32 antibodies have no affinity to rat (99-126)ANP, which differs from human (99-126)ANP by only one amino acid on position 110 (see figure I−3). Knowing that methionine on position 110 of human (99-126)ANP is easily oxidized to the methionine sulfoxide form and that the antibodies used are highly specific for this methionine, a non-oxidized tracer for these antibodies had to be developed.

Iodination

In order to develop a suitable tracer, two methods of iodination were compared: a method in which a mild oxidizing agent, namely Protag, was used and a second method using chloramine−T, which is a more aggressive agent. Figure II−1 shows the elution profiles of both iodination procedures. With the

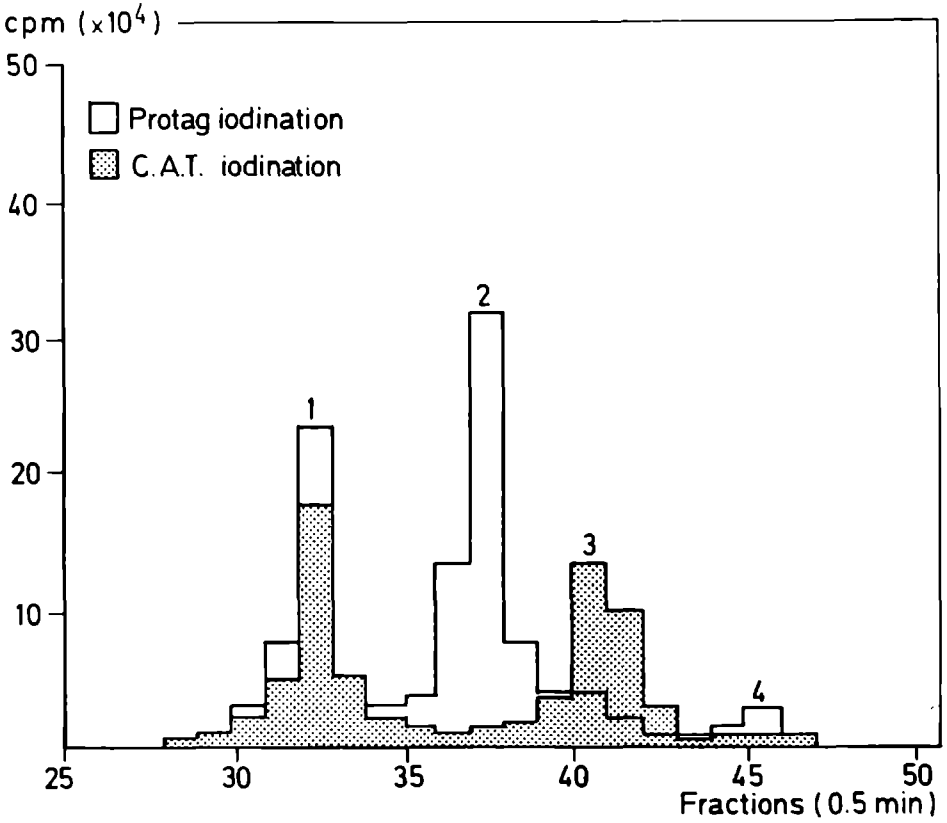

Figure II-1. Elution profile of iodinated (99-126)hANP tracer using two different iodination procedures, namely the Protag method and the chloramine-T (C.A.T.) method.

Protag method, two major (peaks 1 and 2) and two minor peaks (peaks 3 and 4) were observed while with the chloramine–T (C.A.T.) procedure, only two major peaks (peaks 1 and 3) were found. As chloramine–T is a more aggressive method, thus causing more oxidation, we wondered whether peak 1 and peak 3 could be the oxidized forms of peak 2 and peak 4, respectively. Therefore we oxidized fraction 37 (peak 2) and fraction 46 (peak 4) obtained from the Protag iodination with H_2O_2. This resulted in a shift on the HPLC profile to the retention times of peaks 1 and 3, respectively, thereby confirming our hypothesis. Thus peak 1 corresponds to monoiodotyrosyl-(99-126)hANP-methionine sulfoxide, peak 2 to monoiodotyrosyl-(99-126)hANP, peak 3 to diiodotyrosyl-(99-126)hANP-methionine sulfoxide and peak 4 to diiodotyrosyl-(99-126)hANP. Good and reproducible binding was observed with fraction 37 (peak 2, corresponding to the non-oxidized monoiodotyrosyl-(99-126)hANP form). When the tracer of this fraction was diluted, aliquoted and lyophilized it was stable for at least 6 weeks.

As a low binding of the S–32 antibody was observed with the tracers obtained from peaks 1 and 3 which correspond to the oxidized forms of the iodinated (99-126)hANP tracer, it can be assumed that this antibody is more specific for the non-oxidized form of (99-126)hANP. No difference in binding was observed between the oxidized and the non-oxidized tracers when the antibody of Peninsula was used (figure II–2).

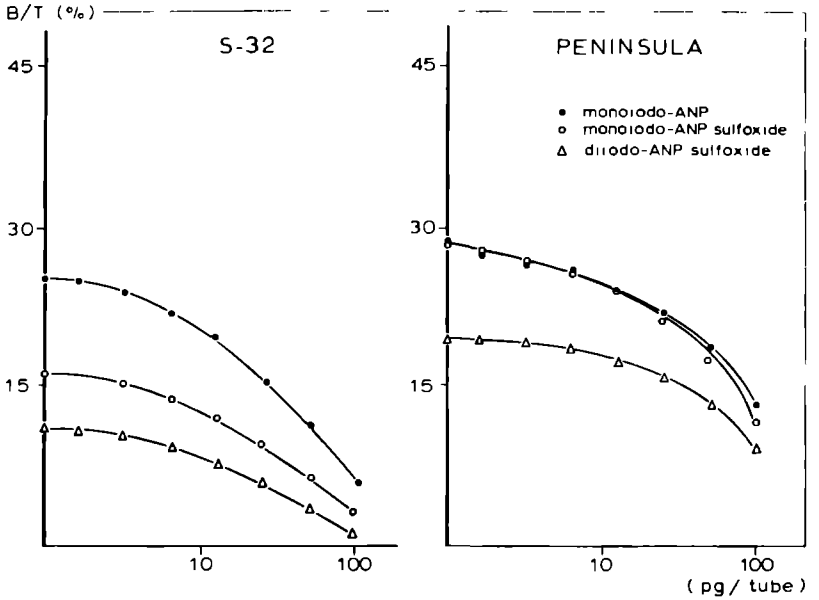

Figure II-2. Various standard curves using both the oxidized and the non-oxidized (99-126)hANP tracers with the S-32 as well as the Peninsula antibody.

Extraction procedure and radioimmunoassay

The extraction procedure is summarized in table II–2. When the eluate of the Seppak C–18 column is air-dried conversion of ANP to the oxidized form might occur, which would then be recognized to a minor extent by the S–32 antibody. Figure II–3 shows ANP values of pure (99-126)hANP standard measured directly in the radioimmunoassay (first column). Air-drying of pure standard in elution solvent resulted in a lowering of immunoreactivity (second column), confirming our hypothesis. However, air-drying of standard added to plasma extracts (third column) or to plasma (fourth column) did not significantly lower ANP immunoreactivity. This implies that plasma exerts a protective effect on the oxidation of ANP. Furthermore instead of air-drying a nitrogen stream is used routinely to minimize oxidation of ANP during the evaporation procedure.

A typical standard curve of the radio-immunoassay is shown in figure II–4.

Table II-2. Flow diagram of extraction procedure and radioimmunoassay.

Collect blood samples (3 ml) in EDTA-tubes on crushed ice
  Centrifuge within 1 hour and store in aliquots containing
  200 KIU aprotinin/ml plasma at -80°C
Extraction on Seppak C-18 cartridges
  Subsequently pretreat with:
  - 5 ml 4 % acetic acid in 86 % ethanol
  - 5 ml methanol
  - 5 ml distilled water
  - 5 ml 4 % acetic acid
Acidify 1.0 ml plasma with 3 ml 4 % acetic acid and apply the
  acidified plasma to the cartridge
Wash twice with 3 ml distilled water
Elute with 3 ml 4 % acetic acid in 86 % ethanol
Evaporate under a stream of nitrogen during 1 hour at 37°C
Add 1 ml of ethanol 100 %, mix and evaporate to dryness
Dissolve in 500 µl RIA-buffer
Radioimmunoassay in polystyrene tubes:
  - take 100 µl plasma extract or standard
  - add 200 µl antiserum
  - mix and cover tubes with parafilm
  - incubate 18 hours at 4°C
  - add 200 µl tracer and mix well
  - cover tubes with parafilm
  - incubate 24 hours at 4°C
  - add 1 ml sheep antiserum/PEG at 20°C and mix
  - incubate for 30 minutes at 20°C
  - centrifuge 30 minutes at 20°C
  - decant supernatant and count pellet

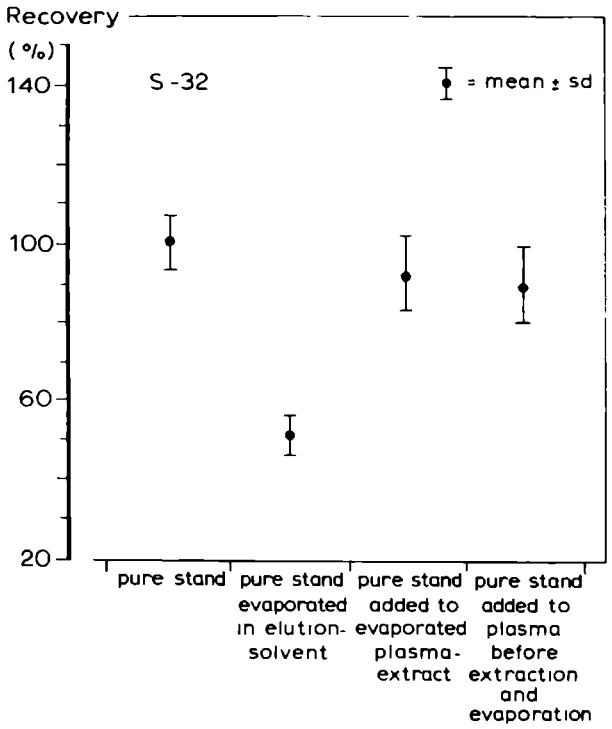

Figure II-3. The effect of evaporation on ANP immunoreactive levels.

The initial binding as determined in 6 consecutive assays was 28 ± 0.5 % (mean ± SD). In these assays a nonspecific binding was obtained of 4.4 ± 1.1 %. The detection limit (defined as B_o - 3 x SD) was 0.8 ± 0.2 pg/tube, corresponding to 4 pg/ml plasma when 1.0 ml plasma was extracted. The 50 % intercept was at 9 pg/tube, with a working range from 0.8 to 100 pg/tube. ANP levels as assessed when 1.0, 0.50 and 0.25 ml of plasma were extracted are shown in table II–3. These results prove that this assay gives a good parallelism. The recovery of synthetic (99-126)hANP added to plasma is constant (95 %) in the range from 25 to 149 pg/ml (table II–4). The intra- and inter-assay coefficients of variation were 8.6 % (n = 7) at a level of 12.6 pg/ml and 11.6 % (n = 6) at a level of 21.7 pg/ml, respectively.

Table II-3. Parallelism of the extraction method (ANP, pg/ml)

Sample no.	1.00	0.50	0.25
	milliliters used for extraction		
1	21.8	27.1	23.4
2	16.6	17.3	18.0
3	15.7	14.0	14.7
4	25.0	26.6	27.6
5	43.1	42.2	41.6
6	46.2	47.4	45.2
7	32.3	32.0	35.2

Table II-4. Recovery of the extraction method (ANP, pg/ml and percentage of recovery)

pg ANP added to different plasmas	ANP (pg/ml) (and % ANP recovered)			
	1	2	3	4
0	46.2	32.3	25.0	43.1
12.5	56.3 (96)	41.7 (93)	34.1 (91)	55.3 (99)
25.0	70.1 (98)	56.2 (98)	46.4 (93)	63.1 (93)
50.0	95.0 (99)	74.1 (90)	66.3 (88)	81.2 (87)
100.0	142.7 (98)	128.3 (97)	121.4 (97)	148.9 (104)

Recovery: mean ± SD = 95 ± 5 % (range 87 to 104 %)

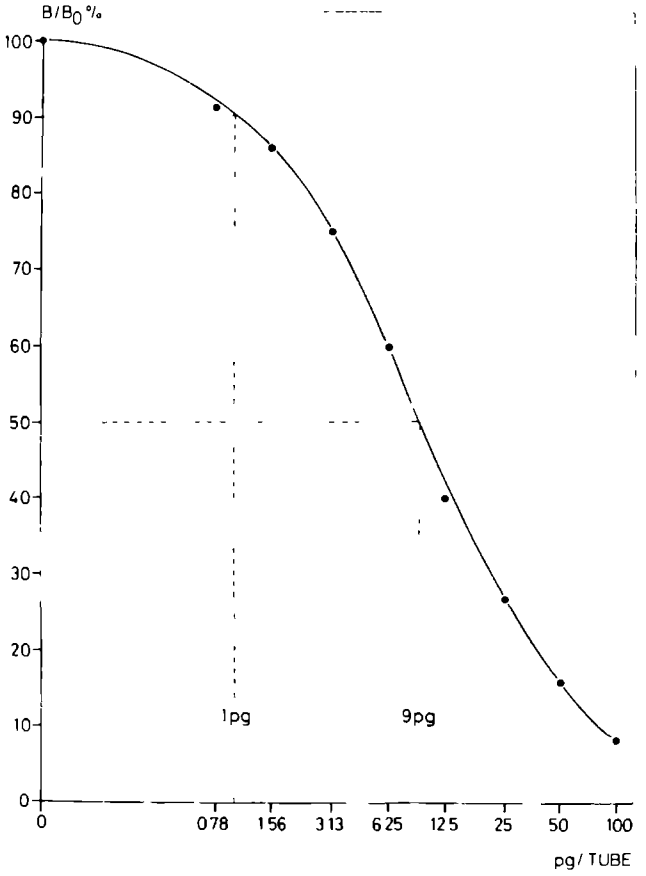

Figure II-4. Standard curve of the extraction method.

Using this extraction method, ANP levels were measured in 66 healthy individuals without a history of cardiovascular, renal or endocrine disorders. Blood was obtained between 9^{30} and 10^{00} a.m. after 30 minutes in the sitting position and after at least 3 hours of fasting. ANP values in these subjects ranged from 8.3 to 86.8 pg/ml (mean ± SD = 35.3 ± 16.2 pg/ml).

In summary, this chapter describes the development and validation of a sensitive and specific method for measurement of ANP concentrations in human blood. This assay, being able to detect both elevated ANP levels and ANP levels below the normal range, can be used for routine measurements of ANP concentrations in man.

Chapter 2.2

Direct method

Introduction

As outlined in the previous chapter, the methods to assess ANP levels in plasma can be divided into direct assays (2–5) as opposed to assays using an extraction step (6–15). In chapter 2.1 an extraction method for measurement of ANP is described. This method, though giving reliable results, is quite laborious and costly. Therefore a simple, easy to perform direct assay for ANP was developed. The results obtained with this direct assay are compared to those obtained with the extraction method, including the effect of exercise stimulating cardiac ANP secretion.

Materials and methods

Materials

Synthetic (99-126)hANP (for constructing standard curve and iodination) was purchased from Peninsula Laboratories Inc, England; aprotinin (Trasylol) was from Bayer, Leverküsen, FRG. Sheep IgG, Bovine Serum Albumin (BSA Cohn Fraction V) and Triton X–100 were from Sigma Chemicals Corp, St. Louis, MO, USA; PEG-6000, EDTA, H_3BO_3 and NaOH were obtained from Merck, Darmstadt, FRG. All reagents were of analytical grade, except where mentioned otherwise. The extraction method was performed as described in chapter 2.1.

Blood sampling

Blood samples were collected by venepuncture in EDTA vacuum tubes, kept on ice, centrifuged within one hour (1500 g, 4°C, 10 minutes) and stored in aliquots containing 200 KIU of aprotinin/ml plasma at −80°C until used.

Direct method

For the direct assay, the antibody S–31 was used. For characterization of the antibody, see table II–1. One hundred microlitres of plasma or standard (in RIA-buffer with 6 % human serum albumin) was mixed with 200 µl of sheep anti-ANP serum (S–31, final dilution in RIA-buffer 1:1.75 x 10^6) at 4°C in poly-

styrene tubes. After an incubation period of 8 hours at 4°C, 200 µl of tracer solution (9500 dpm of non-oxidized monoiodotyrosyl tracer obtained with the Protag iodination method) was added and the mixture was incubated for 24 hours at 4°C. Separation of bound and free hormone was achieved by adding 1.0 ml horse anti-sheep precipitating complex (1 µl normal sheep serum, 15 µl horse anti-sheep-IgG serum and 30 mg PEG-6000 per ml RIA-buffer) at room temperature (20°C) for 30 minutes. After centrifuging for 30 minutes at 1500 g (20°C) the tubes were decanted and the bound fraction was counted in a gamma-counter. Recovery of ANP was studied after addition of 62.5, 125, 250 and 500 pg/ml plasma of synthetic (99-126)hANP to 4 different plasma samples. Parallelism was studied by assaying 5 dilutions of 6 different plasma samples.

Exercise

The effect of exercise on a bicycle ergometer on ANP levels was studied on three males and one female (aged 20 to 28 years). Blood samples were collected before exercise, after a five minutes period of exercise (at 75 % of maximum workload, as determined via a previous submaximal exercise test) and after an one-hour rest period. ANP was measured both with the direct assay and with the extraction assay. Plasma adrenaline (Adr) and noradrenaline (NAdr) were determined as described previously (20).

Statistical analysis

Statistical analysis was performed using the Spearman rank correlation test. Significance was assessed at the $\alpha=0.05$ level. Data are expressed as mean ± SD, except where mentioned otherwise.

Results

Radioimmunoassay

The cross-reactivities of the antibody S−31 are presented in table II−1. As the S−31 antibody is specific for the non-oxidized form of methionine on position 110 of (99-126)hANP, a non-oxidized monoiodotyrosyl-(99-126)hANP tracer as obtained with the Protag iodination method was used in the assay. In figure II−5 a typical standard curve for the direct assay is shown. The detection limit (defined as B_o - 3 x SEM) was 1.34 ± 0.10 pg/tube (mean ± SEM, for 10 consecutive assays), corresponding to 13.4 pg/ml plasma. The 50 % intercept was at 220 pg/ml, giving a working range of approximately 13 to 1000 pg/ml. Five dilutions from 6 plasma samples (ANP values ranging from 45 to 709 pg/ml)

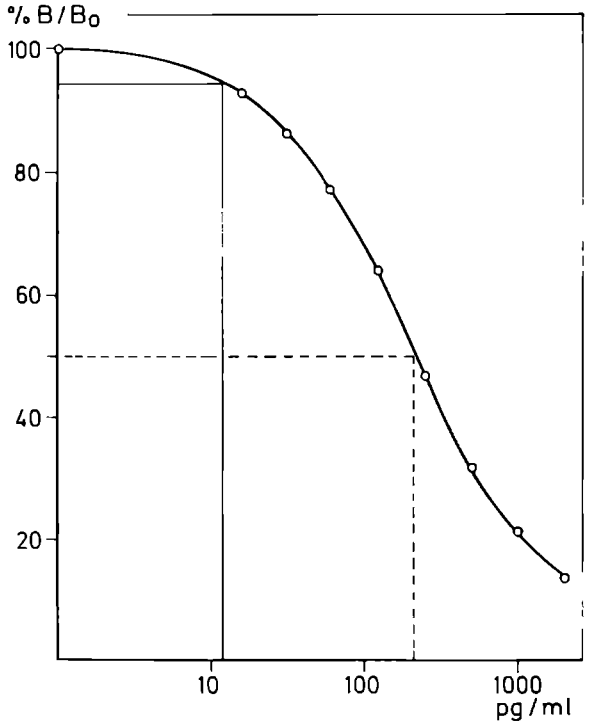

Figure II-5. Standard curve of the direct method.

showed good parallelism (table II–5). The intra- and inter-assay coefficients of variation were 3.1 % (n = 6) and 5.5 % (n = 6), respectively, at an endogenous level of 125 pg/ml. Recovery, as determined by addition of synthetic (99-126)hANP ranged from 90 % to 115 % (mean ± SD = 99 ± 7 %, table II–6).

Plasma ANP values in 41 normal persons with a non-standardized sodium intake were 59 ± 25 pg/ml (mean ± SD, n = 41, range 15 to 111 pg/ml). These values are in range with those reported by other workers in the field (2–15). Thirty-three samples from both normal persons (NP) and patients with congestive heart failure (CHF) were measured using both the direct and the extraction method. The results are shown in figure II–6. The direct assay and the extraction method correlated well; correlation coefficient r = 0.60 ($p<0.01$). However, when only the values of the normal individuals (extraction values ranging from 14 to 70 pg/ml) were studied, the correlation coefficient was 0.34 (p = NS, figure II–6 inset). This discrepancy cannot be ascribed to the use of two different antibodies, as will be shown in chapter 2.3.

Table II-5. Parallelism of the direct method (ANP, pg/ml)

Sample no.	100	80	60	40	20
	microlitres used for radioimmunoassay				
1	493	490	538	517	515
2	709	698	740	850	840
3	411	428	463	408	560
4	68	66	72	78	45
5	45	40	38	43	-
6	104	88	84	101	106

Table II-6. Recovery of the direct method

pg ANP added to different plasmas	ANP (pg/ml) (and % ANP recovered)			
	1	2	3	4
0	27	25	17	15
62.5	83 (90)	83 (93)	73 (90)	82 (107)
125	145 (94)	149 (99)	137 (96)	143 (102)
250	289 (105)	313 (115)	264 (99)	256 (96)
500	574 (109)	499 (95)	541 (105)	480 (93)

Recovery: mean ± SD = 99 ± 7 % (range 90 to 115 %)

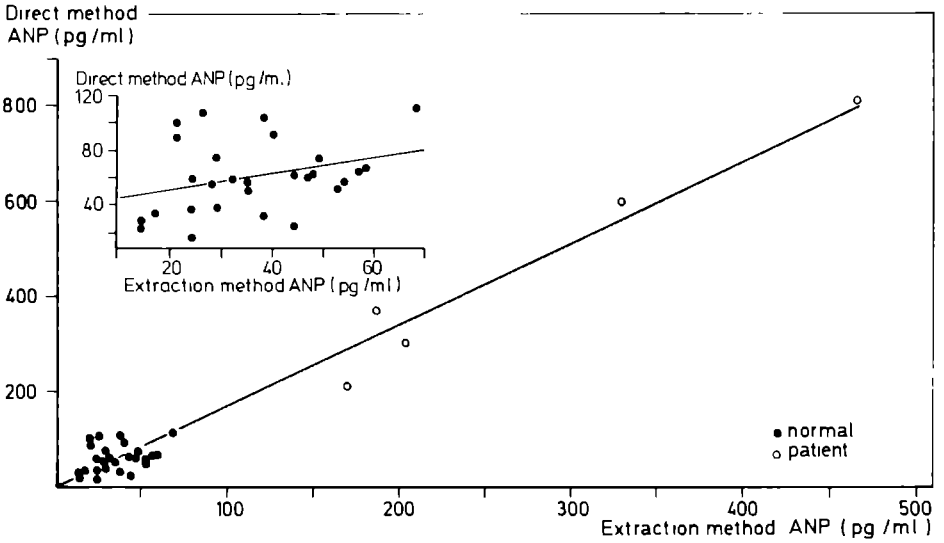

Figure II-6. ANP values of both normal persons and patients with congestive heart failure, measured directly as well as after extraction. The Spearman correlation coefficient was 0.60 ($p<0.01$). Inset: ANP values of normal persons only (correlation-coefficient 0.34, p = NS).

Exercise

ANP values, both measured with and without extraction, increased during exercise on the bicycle ergometer. One hour after exercise, ANP levels were not different from levels prior to exercise (figure II–7). ANP extraction values correlated excellently with values measured with the direct assay ($r = 0.89$, $p<0.001$). Both adrenaline and noradrenaline levels were higher during exercise (table II–7).

Discussion

The correlation of the results from the previously described extraction assay (see chapter 2.1) and the direct assay under discussion was good, with one exception. When ANP values in normal subjects and patients with CHF were pooled (extraction values ranging from 14 to 560 pg/ml), linear regression analysis revealed a good correlation ($p<0.01$). In contrast, no statistically significant

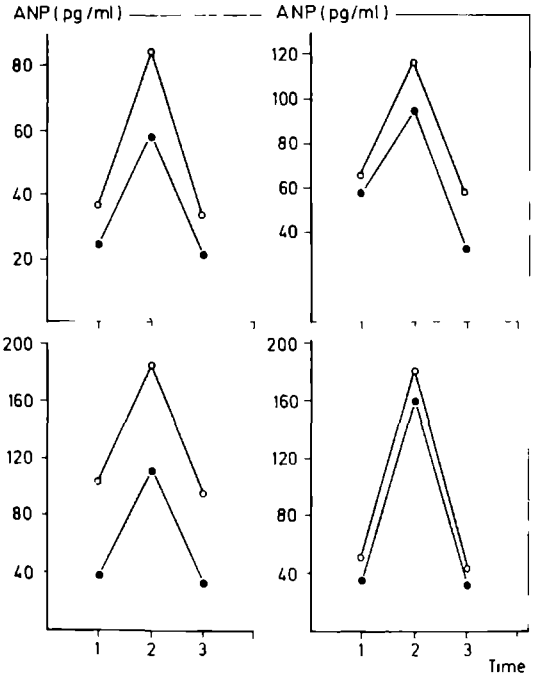

Figure II-7. ANP values of four normal persons before (time 1), during (time 2) and after (time 3) exercise, measured both with the direct assay (○—○) and with the extraction assay (●—●).

Table II-7. Exercise-test on bicycle ergometer

	Pre exercise	During exercise	Post exercise
ANPe (pg/ml)	38.6 ± 14.0	106.3 ± 42.1	29.9 ± 5.7
ANPd (pg/ml)	64 ± 29	141 ± 49	58 ± 28
NAdr (nmol/l)	2.28 ± 0.42	9.78 ± 3.67	2.50 ± 0.48
Adr (nmol/l)	0.17 ± 0.03	0.81 ± 0.18	0.09 ± 0.01

Results of ANP, measured directly (ANPd) and after extraction (ANPe), adrenaline (Adr) and noradrenaline (NAdr). Results are expressed as mean ± SD.

correlation was found when only ANP values in normal subjects (extraction values ranging from 14 to 70 pg/ml) were considered. This discrepancy is in agreement with findings reported by other workers that the correlation of direct results and extraction results decreases at concentrations lower than 100 to 120 pg/ml (13,21–23). Poole *et al.* (24) compared the results of 23 different laboratories who measured ANP values in the same three plasma samples containing low, medium and high ANP immunoreactivity. Although ANP values among the different laboratories varied considerably, all laboratories using an extraction method consistently ranked the three plasma samples in the right sequence ,whereas the laboratories using a direct method did not. Evidently, in unextracted plasma there are factors whose influence becomes the greater as measured ANP concentrations decrease, and which render direct values in the normal range meaningless.

Nevertheless, the direct assay for ANP fulfills some of the criteria of a valid method, such as the parallelism between serial dilutions of plasma and standard curve and the recovery curves of ANP added to plasma. Also, ANP values rose consistently during exercise, in accordance with ANP values measured in extracted plasma (25–27). Other workers using a direct assay have found high levels of ANP in patients with cardiac disease, liver cirrhosis and renal disease (21,28–30). ANP values rose during exercise (28,30), saline infusion (21) and fell after haemodialysis in end-stage renal failure (2,21,28).

Part of the discrepancy in ANP immunoreactivity observed between direct and extraction methods may be explained by the existence of a high molecular weight component which is not retained on the Seppak C–18 column (21,31). This high molecular weight component is clearly different from (1-126)ANP and possesses ANP-binding capacities. Possibly this high molecular weight component represents a binding protein for ANP. The ANP value obtained using the direct assay then represents total ANP immunoreactivity while the ANP value obtained using the extraction method represents the free ANP concentration (32).

In conclusion, direct measurements of ANP, being simple and easy to perform, could well be of value to detect high ANP levels in patients and to follow trends in ANP concentrations in individual subjects. The more laborious extraction method however is necessary for accurate measurements of ANP and to detect small differences in ANP values.

Chapter 2.3

Validation of the antibody

Introduction

To date, various investigators have described methods for measurement of ANP in human plasma. With these methods, widely different normal ANP concentrations have been reported. This may be due to differences of method or technique, or to other factors. Evidence is accumulating that besides the 28 amino acids polypeptide (99-126)hANP other immunoreactive ANP-related forms circulate in human blood (33,34). Different cross-reactivities of the chosen antibody for these as yet unidentified components could partly explain the different outcomes. The antibodies S−31 and S−32 possess different cross-reactivities to several ANP-fragments, as compared to those of the commercially available antibody from Peninsula. To validate the use of the S−32 antibody for the extraction method, plasma extracts were assayed using both the S−32 and the Peninsula antibodies. Furthermore, as the S−32 antibody is used routinely in the extraction method and the S−31 antibody in the direct method, the interchangeability of these two antibodies was studied.

Materials and methods

For the comparison of the S−32 antibody and the Peninsula antibody, 1.0 ml of 56 plasma samples was extracted using the procedure described in chapter 2.1. The plasma-extracts were dissolved in 500 μl RIA-buffer and subsequently radioimmunoassayed using both the S−32 and the Peninsula antibodies. For comparison of the S−31 and the S−32 antibodies in the extraction method, both antibodies were applied to 17 plasma extracts. Both antibodies were also applied on 30 plasma samples using the direct assay.

Statistical analysis

For comparison of ANP values obtained using the different antibodies the paired t-test (Student) was used. Furthermore the Spearman rank correlation test was used for determination of the correlation coefficient. Significance was assessed at the α=0.05 level.

Results and discussion

Figure II–8 shows ANP levels as determined with the S–32 antibody and the antibody from Peninsula on 56 plasma extracts (S–32 values ranging from 8 to 94 pg/ml). An excellent correlation between these two antibodies was observed, $r = 0.88$, $p<0.001$. When the Peninsula antibody was used generally higher ANP values were found as compared to those found when using the S–32 antibody ($p<0.01$). Linear regression analysis resulted in the following equation (mean ± SEM): S–32 value = 0.81 (± 0.06, $p<0.001$) x Peninsula-value - 3.73 (± 3.20, p = NS) pg/ml. The observed discrepancy can be explained by different cross-reactivities of the S–32 and the Peninsula antibodies towards various fragments of (99-126)hANP as displayed in table II–1. As high pressure liquid chromatography analysis indicates the existence of at least three distinct ANP-related components in human blood (chapter 2.4) different crossreactivities of the two antibodies towards these components might easily result in different ANP values.

In figure II–9 the results of both the S–31 and the S–32 antibodies on 17 plasma extracts (S–32 values ranging from 13 to 62 pg/ml) are shown, while in figure II–10 the results of both antibodies on 30 plasma samples in the direct

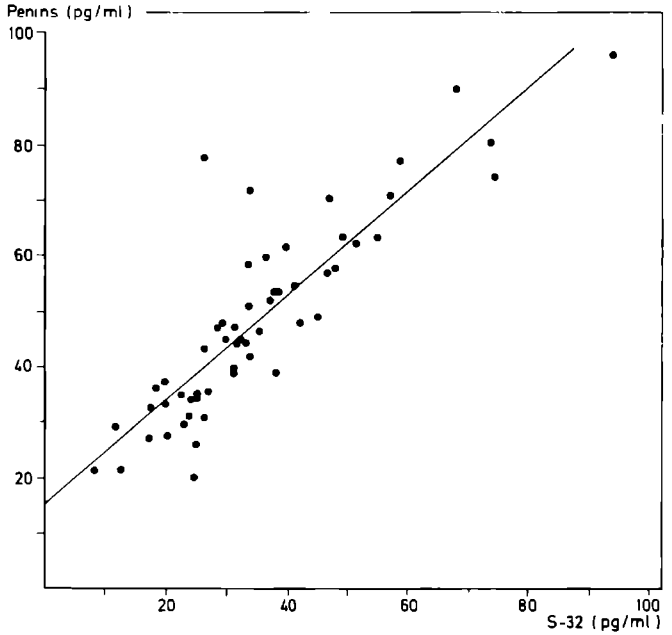

Figure II-8. ANP values and regression line obtained when both the S-32 and the Peninsula antibodies were used for determination of ANP concentrations in 56 plasma extracts.

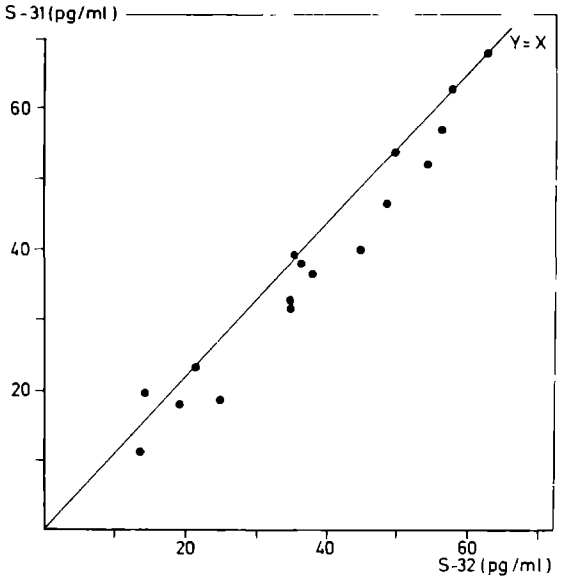

Figure II-9. Results obtained when both the S-31 and the S-32 antibodies were used in the extraction method (n = 17).

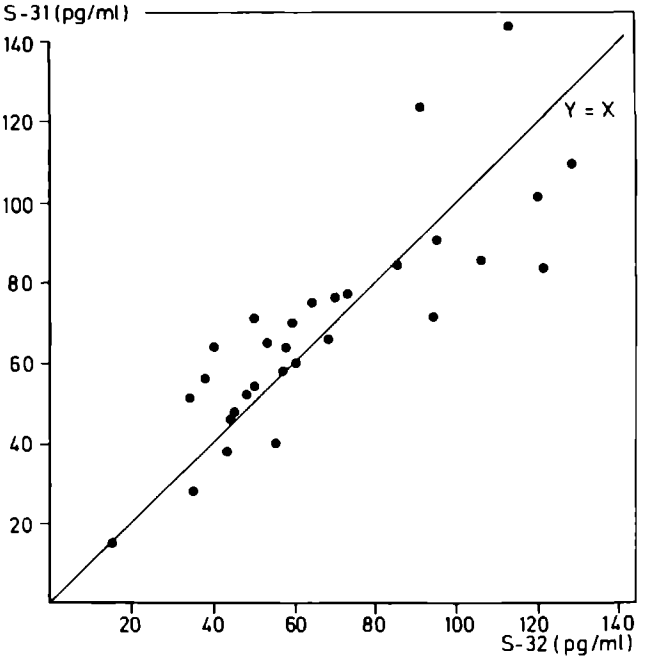

Figure II-10. Results obtained when both the S-31 and the S-32 antibodies were used in the direct method (n = 30).

assay (S−31 values ranging from 15 to 143 pg/ml) are shown. Values obtained using both antibodies correlated well both when the extraction method was used ($r = 0.97$, $p<0.01$) and when the direct method was used ($r = 0.87$, $p<0.01$). Use of both antibodies resulted in similar ANP values in either assay (paired t-test, extraction method: $t = -0.22$, $p = 0.83$, direct method: $t = 0.33$, $p = 0.75$). These results therefore cannot explain the discrepancy between ANP values obtained with the extraction method (S−32 antibody) and with the direct method (S−31 antibody) on the same plasma samples from normal persons, as described in chapter 2.2. These results also demonstrate the possibility of interchanging both antibodies for the two methods to determine ANP concentrations described in this thesis. Mainly for reasons of economy the S−32 antibody is used routinely in the extraction method while the S−31 antibody is used routinely in the direct assay.

The correlation observed between the S−32 antibody and a widely used commercially available antibody from Peninsula justifies the use of the former antibody for use in routine measurements of ANP in human blood.

Chapter 2.4

Immunoreactivity of ANP in human plasma

Introduction

The exact molecular forms of atrial natriuretic peptide in human plasma are not yet known. One method to investigate these molecular forms is separation of ANP-immunoreactivity on a high pressure liquid chromatography (HPLC) column.

Nakaoka *et al.* (35) using an extraction method, found elevated levels of ANP in patients with congestive heart failure. This finding was soon confirmed by various other workers (36,37). However, it is questionable whether these elevated levels of immunoreactive ANP can be attributed solely to the (99-126)hANP form, or that other immunoreactive forms are measured also.

To investigate this possibility plasma from 11 patients with congestive heart failure (CHF) and plasma from 5 normal persons (NP) were applied to HPLC-analysis. As besides (99-126)hANP two other immunoreactive forms were detected in human blood, the functional relevance of these immunoreactive forms was studied by HPLC-analysis of plasma obtained both in the basal state and during stimulated cardiac ANP release.

Materials and methods

Blood was collected into EDTA tubes on ice, and centrifuged immediately (10 minutes, 1500 g, 4°C). Prior to HPLC-analysis, the plasma was extracted on Sep-pak silica C−18 cartridges (Waters Associates, Milford, MA, USA), subsequently eluted using an acetic acid (96 %)-ethanol(86 %) (4:96 v/v) solution, evaporated under a nitrogen stream and dissolved in RIA-buffer (pH 8.4, 0.2 mol/l sodium borate buffer). HPLC-analysis was performed using a Supelco C-18 (5 μm)-column (25 cm) with a flowrate of 2 ml/min. The initial solvent composition was acetonitrile-distilled water-TFA (20:80:0.1 v/v/v). A linear gradient was used from 20 to 34 % acetonitrile at 30 minutes, and to 80 % acetonitrile at 40 minutes after injection of 200 μl extract. Fractions were collected each minute. (99-126)hANP eluted at 28 % acetonitrile (fraction 16). Subsequently, these fractions were radioimmunoassayed using two different antibodies: the S−32

antibody and the Peninsula antibody. Cross-reactivities of these two antibodies are displayed in table II−1.

The plasma samples were assayed both directly and after extraction, measuring total immunoreactivity, and after application to the HPLC-column. Plasma for HPLC-analysis was obtained from 11 patients with chronic congestive heart failure (Killip classification score III and IV (38)) due to various diseases, and from 5 normal persons without any signs of cardiovascular, renal or endocrine disorders.

To study the influence of cardiac ANP secretion on the immunoreactive ANP-related forms blood was obtained from 5 healthy young persons before and during exercise at 90 % of maximal capacity on a bicycle ergometer. Also, from 1 healthy young person blood was obtained before and after stimulation of ANP by volume loading (750 ml 0.9 % NaCl during 60 minutes). The study protocol of the exercise test is described in detail in chapter 3.6 and the volume loading study is described in chapter 4.4.

To study the correlation between the various methods and the two antibodies, the Spearman rank correlation test was used. Differences in contents of peaks A, B and C expressed as percentages of total ANP immunoreactivity measured in peaks A, B and C on the HPLC-profile were studied using the Wilcoxon two-sample test. Differences between values obtained with the Peninsula antibody and with the S-32 antibody were investigated using the Wilcoxon signed rank test. Significance was assessed at the $\alpha=0.05$ level. Data are expressed as mean ± SD, except where mentioned otherwise.

Results

After HPLC-analysis, a large amount of immunoreactive material did not elute on the position of (99-126)hANP (peak C, fractions 15 to 17). Figure II−11 shows typical HPLC-profile patterns of plasmas from a NP and a CHF person. Both types of plasma showed besides the major (99-126)hANP peak (peak C), two minor peaks preceding peak C. Peak A eluted at positions 8, 9 and 10 while peak B eluted at positions 12, 13 and 14. These three peaks were observed both when the S−32 antibody was used and when the Peninsula antibody was used. When the content of peak C, measured with the S−32 antibody, was expressed as percentage of the total ANP immunoreactivity measured in peaks A, B and C, peak C contained 46 to 73 % of total immunoreactivity in the CHF plasma, and 40 to 58 % in the plasmas of NP. Using the Peninsula antibody, the comparable ranges were 23 to 60 % and 35 to 49 %, respectively (table II−8). The contents

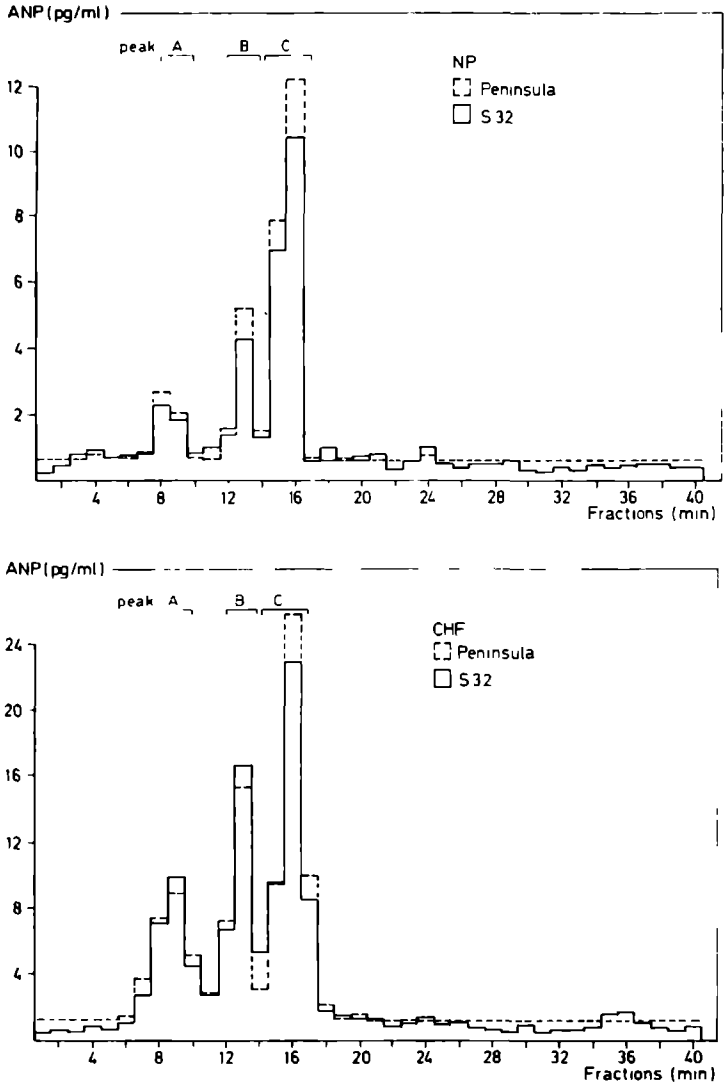

Figure II-11. Representative HPLC profiles of both a normal person (NP, upper panel) and of a patient with congestive heart failure (CHF, lower panel) measured with the S-32 as well as the Peninsula antibody.

of peaks A, B and C did not differ between NP and CHF, neither when using the Peninsula antibody nor when using the S−32 antibody. When the NP and the CHF persons were taken together, the percentage content of peak A as measured with the Peninsula antibody was significantly greater than the

Table II-8. Contents of immunoreactive peaks in the HPLC profile (expressed as percentage of total immunoreactivity of peaks A, B and C) measured with the Peninsula antibody and with the S-32 antibody.

		Mean ± SD (range) %	
		Peninsula	S-32
CHF	peak A	32.3 ± 12.3 (20 - 60)	18.7 ± 6.4 (10 - 29)
	peak B	24.1 ± 5.8 (17 - 34)	22.2 ± 5.2 (14 - 32)
	peak C	43.7 ± 10.6 (23 - 60)	59.2 ± 9.4 (46 - 73)
NP	peak A	26.8 ± 4.7 (21 - 34)	23.1 ± 3.1 (19 - 27)
	peak B	30.1 ± 4.3 (26 - 37)	28.8 ± 4.7 (23 - 35)
	peak C	43.2 ± 5.2 (35 - 49)	48.0 ± 7.6 (40 - 58)

percentage content of peak A as measured with the S−32 antibody. For peak B, these percentages did not differ, while the percentage content of peak C was less for the Peninsula antibody.

Also, the total ANP immunoreactivity after extraction of the plasma was measured. Taken these percentages into account, the (99-126)hANP levels (corresponding to peak C) ranged from 72 to 330 pg/ml in CHF as measured with the S−32 antibody, and from 45 to 380 pg/ml as measured with the Peninsula antibody. The ranges in NP were 12 to 35 pg/ml and 9 to 34 pg/ml, respectively.

When ANP concentrations as measured with the direct assay, the extraction method and as content of peak C on the HPLC-profile were correlated using the S−32 and the Peninsula antibodies, overall results correlated well (table II−9 and figure II−12). Figure II−13 shows representative HPLC-profiles of plasmas obtained both in the basal state and during stimulation (bicycle exercise or volume loading). Under both basal and stimulated conditions, the predominant immunoreactive component as secreted by the heart corresponds to (99-126)hANP.

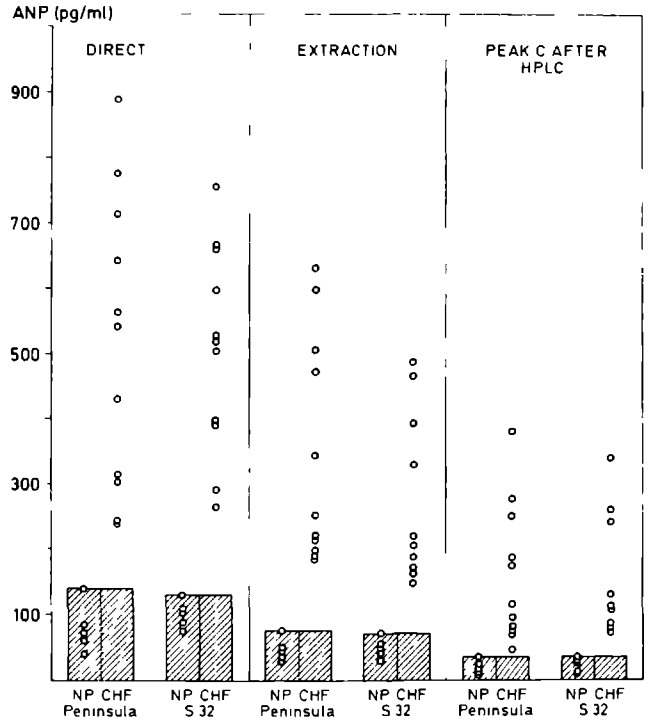

Figure II-12. ANP immunoreactivity measured with the direct assay, the extraction assay, and as content of peak C on the HPLC profile, using both the S-32 and the Peninsula antibodies.

Discussion

Early reports on ANP-immunoreactivity, based on separation of plasma by HPLC-chromatography, indicated that the main immunoreactive form in human plasma corresponds to (99-126)hANP. However, several investigators using various HPLC-techniques observed besides (99-126)hANP also other immunoreactive moieties. These other immunoreactive forms may represent smaller fragments of (99-126)hANP like (103-125)hANP, (102-126)hANP, (103-126)hANP or (105-126)hANP. Also the existence of ß-hANP, (1-126)hANP (the prohormone) and (1-98)hANP has been postulated (33,34,39–41). The differences in techniques used, in specificity of the antibody used and the complexity of the HPLC-technique may explain these discrepancies. In this respect the Peninsula antibody recognized the ANP moiety eluting at peak A of the HPLC-profile to a

Table II-9. Spearman correlation coefficients between ANP values measured with the direct method, the extraction method and as content (pg/ml) of peak C on the HPLC profile using both the S-32 and the Peninsula antibodies. All correlations were highly significant ($p<0.01$).

Spearman r n = 16		Peninsula		
		direct	extraction	HPLC
	direct	0.96	0.76	0.81
S-32	extraction	0.77	0.96	0.95
	HPLC	0.77	0.93	0.97

greater extent than the S−32 antibody. Consequently, the percentage content of peak C recognized by the Peninsula antibody decreased and was less than the percentage content of peak C recognized by the S−32 antibody.

Using HPLC-analysis and amino-acid sequence analysis, Yandle *et al.* (33) found that both (99-126)hANP, (106-126)hANP and a peptide made up of (99-105)hANP and (106-126)hANP circulate in human blood. The results obtained with our HPLC-technique confirm that besides (99-126)hANP, other immunoreactive forms circulate in plasma of both normal persons and patients with congestive heart failure. The existence of these immunoreactive forms cannot be ascribed to the use of the S-32 antibody, as these three peaks on the HPLC-profile were also found when using the commercially available and widely used Peninsula antibody. As both antibodies are specific for ANP or ANP-related components and do not cross-react with other peptide hormones (see table II−1) peak A and peak B probably represent parts of the (1-126)hANP hormone. Various fragments and extended forms of (99-126)ANP are biologically active (42−44). Therefore it is quite probable that the ANP immunoreactive forms representing peaks A and B are also biologically active.

Both volume loading and exercise stimulate cardiac ANP secretion. As shown in this chapter, the predominant form secreted by the heart corresponds to (99-126)hANP. This implies that, although other ANP-related forms also circulate in human blood, the predominant form of ANP-immunoreactivity both basal and in the stimulated state, is (99-126)hANP. Whether the other two unknown

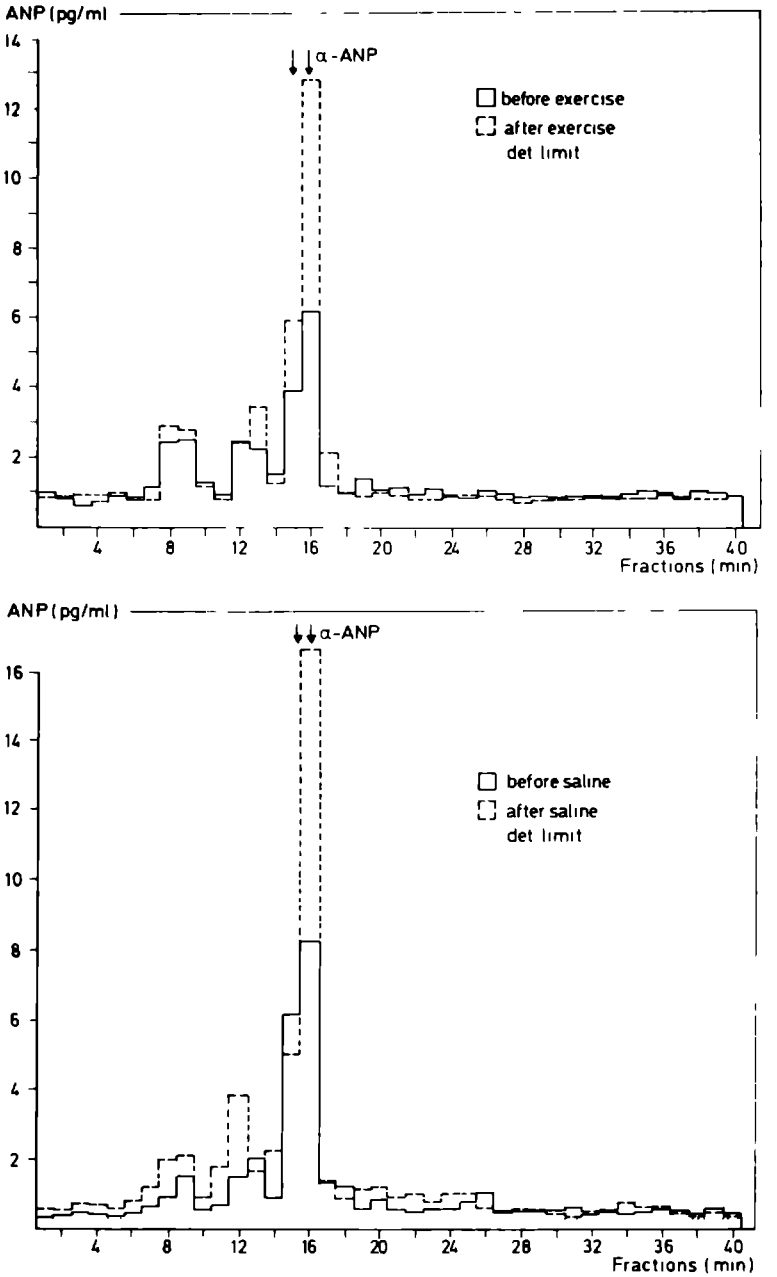

Figure II-13. Representative HPLC profiles of two healthy subjects, both in the basal state and during stimulation by exercise (upper panel) or volume loading (lower panel).

immunoreactive peaks observed in this study may represent some kind of precursor or degradation products of (99-126)hANP and whether they are of physiological importance, remains to be elucidated.

Chapter 2.5

Determination of cGMP concentrations

Introduction

Cyclic guanosine monophosphate (cGMP) acts as the second messenger for the atrial natriuretic peptide hormonal system (45,46). Extracellulair cGMP values correlate well with intracellular cGMP values (47). Therefore determination of cGMP concentrations in plasma reflect the biological effectiveness of ANP (48,49). As combined assessment of both ANP and cGMP concentrations in plasma can provide insight into the functioning of the ANP hormonal system, a method for measurement of cGMP concentrations in human plasma was developed.

Materials and methods

Cyclic GMP was measured by radioimmunoassay following ethanol precipitation of plasma proteins. To correct for procedural losses, 100 μl (10,000 dpm) of ^{3}H-cGMP in ethanol (8-^{3}H-guanosine 3',5'-cyclic monophospate (Amersham International, England) of specific activity 910 GBq/mmol) was dried in borosilicate glass tubes under a stream of nitrogen. The residues were then incubated with 0.5 ml of plasma for 10 minutes. To each sample, 1.0 ml of ethanol (Merck 983, Darmstadt, FRG) was added. The tubes were vortexed and centrifuged at 2000 g for 10 minutes. The supernatants were then decanted and dried at 50°C under a stream of nitrogen. The residues were redissolved in 0.5 ml of assay buffer (50 mM Tris, 4 mM EDTA (Merck, Darmstadt, FRG) of pH 7.5 with 0.625 % (v/v) of beriglobuline (Behringwerke, Marburg, FRG)) and centrifuged at 2000 g for 5 minutes. Of these samples, 200 μl was counted for recovery and two 100 μl aliquots were taken for radioimmunoassay. A standard curve (0 to 100 nmol/l) was set up in duplicate. To each standard, control and unknown, 100 μl (10,000 dpm) of ^{3}H-cGMP, 200 μl of sheep anti-cGMP serum in assay buffer and 200 μl of assay buffer were added. Following overnight incubation at 4°C, antibody bound and free cGMP were separated by addition of 150 μl of dextran coated charcoal (1.6 % w/v of charcoal and 0.2 % w/v of dextran T70 in 50 mM Tris-buffer). After 6 minutes at 4°C the tubes were centrifuged at 2000 g. The supernatants were then decanted and counted for radioactivity. The cGMP an-

tibody was raised in Texel white sheep against cGMP coupled to keyhole limpet haemocyanin.

Results and discussion

After immunization of sheep with cGMP, an antibody with a titre of 1:3000 was obtained and used for routine measurements of cGMP concentrations in human plasma. The cross-reactivity of the antibody with cAMP was 4 %. The cGMP values are corrected for the individual recovery of the plasma sample. The mean (± SD) recovery of 25 plasma samples after ethanol precipitation of

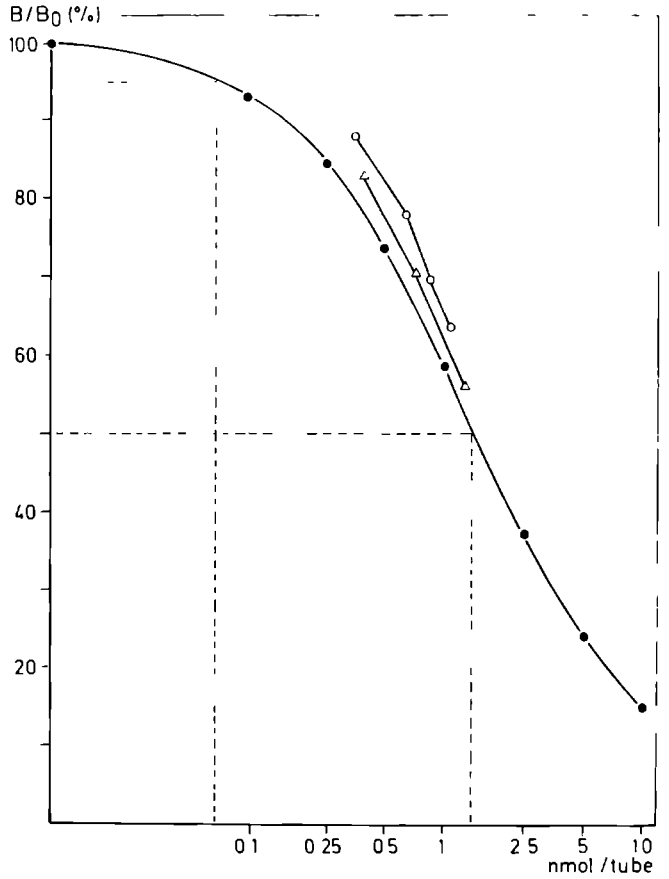

Figure II-14. Standard curve of the radioimmunoassay for cGMP with dilution curves of two different plasma samples.

plasma proteins was 75.4 (± 6.8) %. Dilution of plasma samples showed good parallelism. In figure II−14 a representative standard curve of the radioimmunoassay is shown. The sensitivity of the assay, defined as the least amount of cGMP distinguishable from zero at a 95 % confidence limit was 0.07 pmol per tube, corresponding to 1.0 nmol/l of plasma. The intra- and inter-assay coefficients of variation were 7.1 % (n = 6) and 14.2 % (n = 10), respectively, at a level of 11.5 nmol/l.

These results demonstrate that cGMP concentrations can be measured accurately and reproducably in human plasma. Assessment of cGMP values may be useful to evaluate the biological effect of ANP.

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Part III

INFLUENCE OF VARIOUS PHYSIOLOGICAL AND SAMPLING CONDITIONS ON ANP CONCENTRATIONS

To date, various workers have described methods for measurement of plasma ANP values in humans. Plasma ANP values as obtained with a method using an extraction step of the plasma are generally considered to be reliable (1,2). Yet, even when using the same extraction method and the same antibody, ANP values obtained in various laboratories differ considerably (1). Part of these differences can be ascribed to different blood collection procedures. In addition physiological conditions during the blood sampling procedure may influence cardiac ANP secretion. In this respect one has to consider the position of the patient, a diurnal rhythm, and the influence of exercise. In the following chapters, the influence of the aforementioned factors, along with the influence of venepuncture stress, the menstrual cycle and intra-individual variations on ANP concentrations are analyzed.

Chapter 3.1

Blood sampling and handling of plasma samples

Introduction

No uniform agreement exists regarding the procedure for collection of blood samples for determination of ANP concentrations. Various workers use either EDTA or heparin as an anti-coagulant. Also, the necessity of putting the blood sampling tubes on ice has been questioned. Richards *et al.* (2) reported that, using a direct method, ANP-immunoreactivity varied with the number of platelets present in plasma. Since this number is greatly dependent on the mode of centrifugation, the effect of different modes of centrifugation on plasma ANP levels is described in this chapter. Plasma samples for determination of ANP values are usually not assayed immediately. Before assaying the samples, they are stored at -80°C for periods up to some months. Occasionally, samples have to be assayed once again. Therefore the influences of both storage (for a period up to six months at either -20°C or at -80°C) and repeated thawing of plasma samples before assay were studied.

Methods

Blood collection procedure

To compare various blood sampling conditions, blood samples from two persons were collected into either EDTA, heparin or citrate glass tubes (kept on ice). After centrifugation of the blood sample (1500 g, 10 minutes, 4°C), 200 KIU of aprotinin (Trasylol, Bayer AG, FRG) was added for each milliliter of plasma. Each sample was measured in sixfold.

The analytical stability of ANP was studied by centrifuging blood samples obtained from one person after 0, 1 and 3 hours at either room temperature (20°C) or stored in ice (0°C). Each sample was measured in sixfold. All samples of each person were measured consecutively in the same assay to minimize intra- and inter-assay variations. For determination of ANP, the extraction method with the Peninsula antibody was used.

Centrifugation of blood samples

From three persons, sufficient blood was collected to centrifuge blood samples (9 ml each) respectively 5, 10 and 15 minutes at 1500 g and 15 minutes at 3000 g. Also, following centrifugation for 15 minutes at 1500 g, the aspirated plasma was recentrifuged for 15 minutes at 20,000 g. After centrifugation the plasma columns were aspirated in four portions from the top to the buffy coat (separating the plasma from the red cells). Before storage at −80°C, an aliquot (25 μl) was removed for counting of the platelets. The samples were measured consecutively in the same assay run. Both the extraction method (with the S−32 antibody) and the direct method (with the S−31 antibody) were used for determination of ANP values.

Storage and thawing of plasma samples

The effect of prolonged storage (up to 5 months) at −80°C was studied on one plasma sample, measured repeatedly (14 times) during this period. Also, from two subjects plasma (with aprotinin 200 KIU/ml plasma added) was stored either at −20°C or at −80°C for a period up to six months. The ANP levels (consisting of six measurements each) were compared with the ANP level (six measurements) in plasma assayed within one week (stored at −80°C). To study the influence of thawing, plasma from 3 persons was thawed once (which is the routine procedure), twice or three times before assay. Each plasma sample was measured six times for each of the thawing procedures. Samples of each person, regardless of the thawing procedure, were measured in the same assay run within one week after blood collection. The extraction method using the S−32 antibody was used.

Statistical analysis

Statistical analysis of the various blood collection procedures was performed using the Wilcoxon two-sample test. To correlate the amount of platelets present in the plasma with the corresponding ANP value the Spearman rank correlation test was used. For evaluation of the different modes of centrifugation, one-way analysis of variance followed by the t−test (Student) with Bonferroni's correction was used. Differences in ANP immunoreactivity after storage at −20°C or at −80°C and after several times thawing were studied using one-way analysis of variance followed by the t−test (Student) with Bonferroni's correction. Significance was assessed at the $\alpha=0.05$ level. Data are expressed as mean ± SD, except where mentioned otherwise.

Results

Blood collection procedure

Collection of blood in heparin or citrate glass tubes gave consistently lower values than collection in EDTA glass tubes (table III−1, percentage and p−value as compared to blood sampling in EDTA glass tubes on ice, 0 hours). Obviously, collection in citrate resulted in much greater variations. The amount of immunoreactive ANP decreased when blood was kept either on ice or at room temperature for some time before centrifugation. However, when blood kept on ice was centrifuged within one hour, immunoreactivity remained at 90 % of basal. Based on these results, the following routine procedure was adopted as standard procedure for blood collection: sampling into EDTA-glass tubes kept on ice,

Table III-1. ANP concentrations found after different treatments of blood samples

Subject	Sample treatment	ANP (pg/ml) mean ± SD	%*	p-value
A	EDTA, in ice, 0 h	25.0 ± 1.7	100	-
	Heparin, in ice, 0 h	20.2 ± 1.5	80	p<0.01
	Citrate, in ice, 0 h	12.0 ± 4.7	48	p<0.01
B	EDTA, in ice, 0 h	35.8 ± 2.2	100	-
	Heparin, in ice, 0 h	30.5 ± 2.4	85	p<0.01
	Citrate, in ice, 0 h	25.8 ± 4.7	72	p<0.01
C	EDTA, in ice for:			
	0 h	25.0 ± 1.7	100	-
	1 h	22.5 ± 1.6	90	p<0.05
	3 h	20.8 ± 1.5	83	p<0.01
	EDTA, at RT for:			
	0 h	22.5 ± 1.0	90	p<0.05
	1 h	19.5 ± 1.4	78	p<0.01
	3 h	18.0 ± 1.3	72	p<0.01

Numbers are for six different samples from each subject.
RT: room temperature (20°C). In ice: 0°C. P-value: vs. EDTA in ice, 0 hours (h) for the same person.
* ANP concentration found expressed as % ANP concentration for the same plasma in EDTA, in ice, 0 h.

centrifugation as soon as possible (within one hour) and storage at −80°C before assay.

Centrifugation of blood samples

From each person (n = 3), a blood sample (9 ml) was centrifuged using five

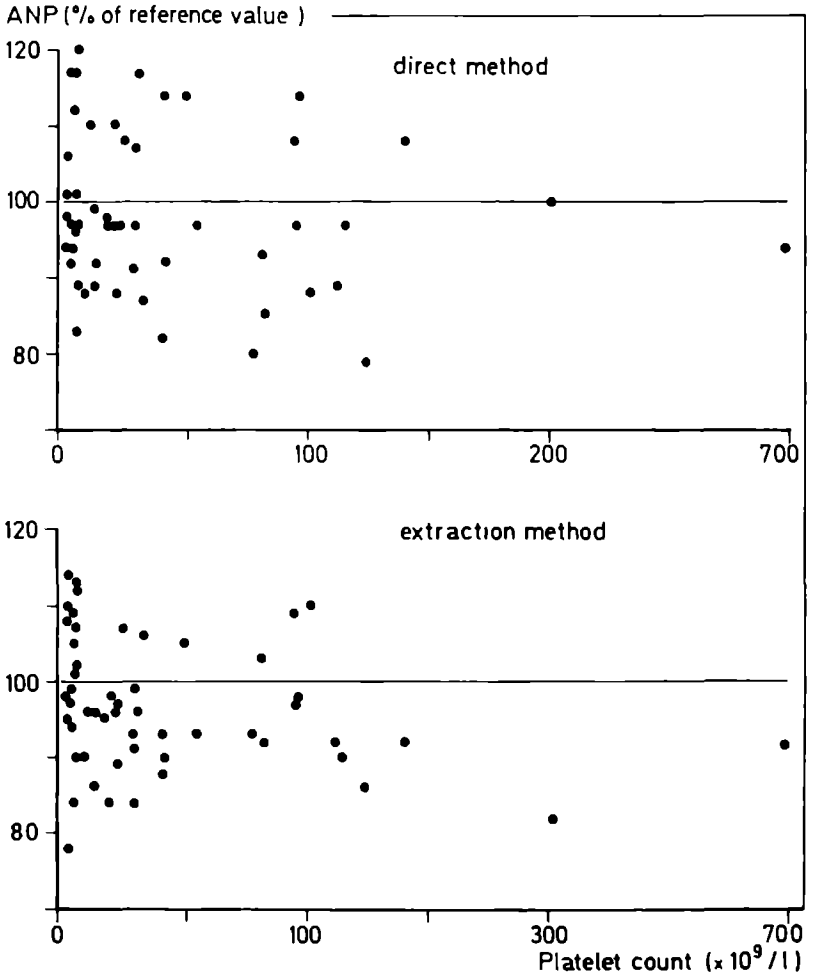

Figure III-1. Relationship between the platelet count in the plasma and the corresponding ANP value, measured with the direct assay (upper panel) and after extraction (lower panel). ANP values are expressed as percentages of the ANP concentrations in plasmas of the same person containing less than 10 x 10^9 platelets/l.

different methods, yielding different amounts of platelets in the plasma. Furthermore, each plasma column was divided into four equal parts with increasing platelet count from the top of the plasma column to the buffy coat. In figure III–1 the ANP values of all individuals are plotted against the corresponding platelet counts. The values that were measured in plasma containing less than 10 x 10^9 platelets/l, were taken as reference value (= 100 %) for each person. No significant correlation was found between platelet count and ANP value as measured with or without extraction. Pooling of the values of the four portions of the plasma columns together, the results of the various procedures of centrifugation are shown in figure III–2. No differences in ANP levels were observed between the various centrifugation procedures.

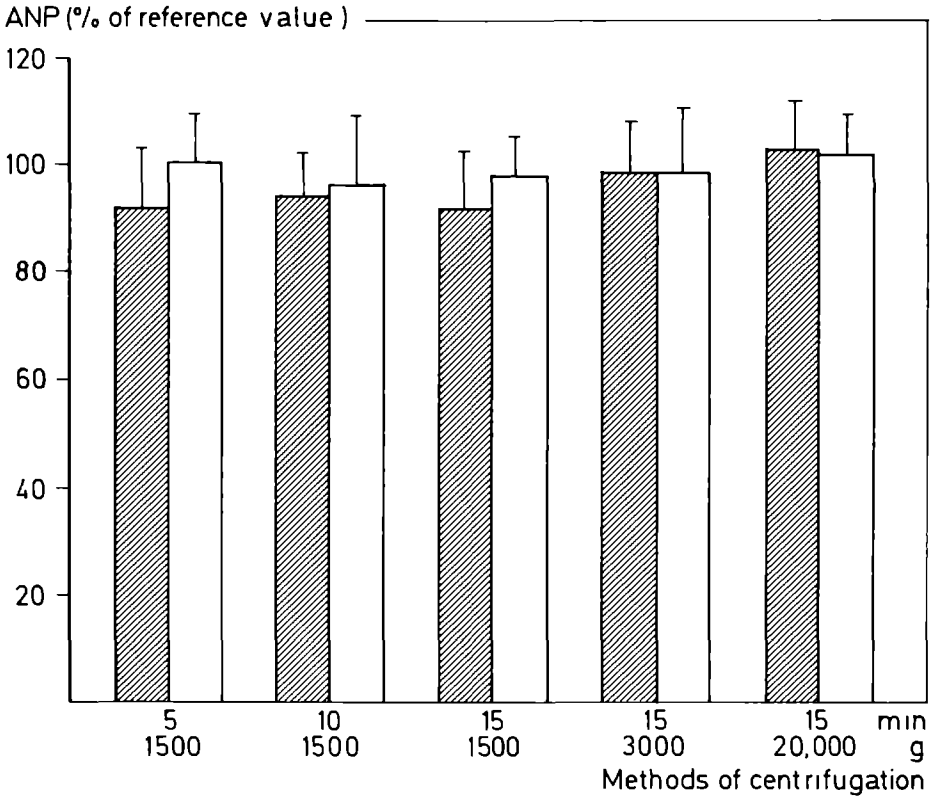

Figure III-2. ANP values found directly (open bars) and after extraction (closed bars) in relation to different centrifugation modes. Bars denote mean ± SD. Values are expressed as percentages of the ANP concentrations in plasmas of the same person containing less than 10 x 10^9 platelets/l.

Storage and thawing of plasma samples

Storage of plasma from one person at −80°C during a period of up to five months did not significantly influence ANP immunoreactivity (figure III-3). However, comparison of storage temperatures revealed that storage at −20°C markedly decreased ANP immunoreactivity as compared to storage at −80°C. Storage at −80°C for a period up to six months did not significantly influence ANP immunoreactivity (figure III−4 and table III−2). In contrast, repeated thawing of plasma prior to extraction and radioimmunoassay markedly decreased the ANP level (figure III−5 and table III−3).

Discussion

Various factors can influence ANP levels, thereby explaining part of the differences in reference values found among various laboratories. An additional factor contributing to these discrepancies in reference values could very well be that blood sampling and handling of the plasma samples differed considerably. As shown by the preceding experiments, the choice of anticoagulant can exert a substantial influence on ANP immunoreactivity. Storage of blood samples, even

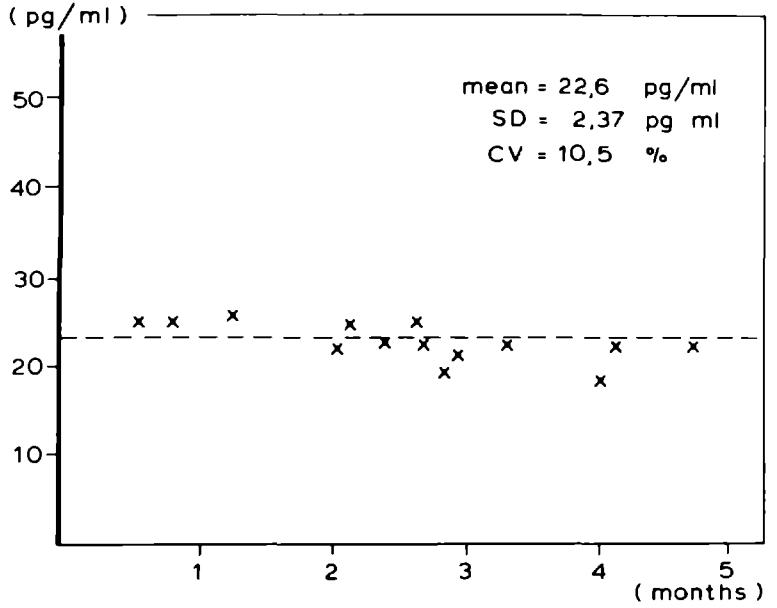

Figure III-3. Stability of ANP immunoreactivity in plasma during storage at −80°C (n = 14).

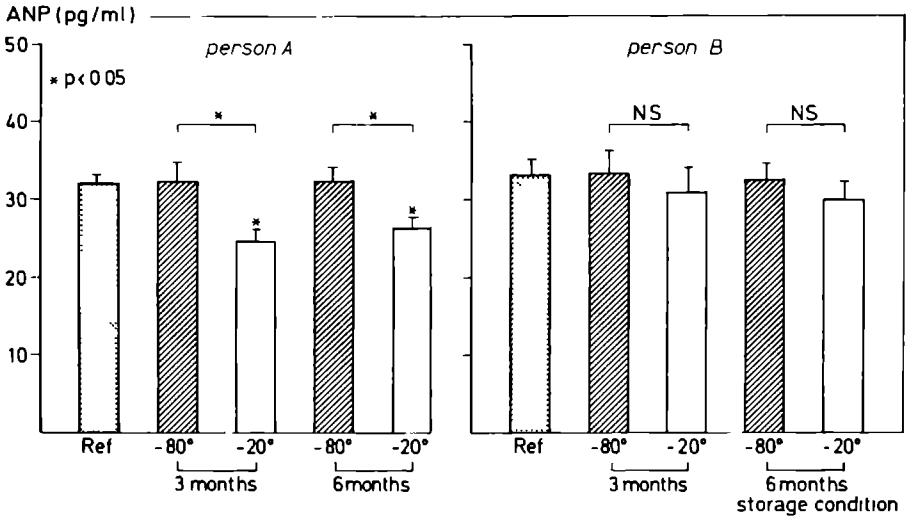

Figure III-4. Storage of plasma at −20°C or at −80°C for a period up to six months. While storage at −20°C decreased ANP levels, storage at −80°C did not influence ANP levels (Ref = reference ANP values in plasma assayed within one week). Values are mean ± SD. p = p−value vs. reference.

Table III-2. Storage of plasma of two subjects at -20°C or at -80°C. Values represent the mean (± SD) of six measurements. Reference = ANP levels in plasma assayed within one week.

Storage	subjects	
	A	B
Reference	32.2 ± 1.0	33.0 ± 2.0
3 months		
-80°C	32.3 ± 2.3	33.6 ± 2.8
-20°C	24.9 ± 1.4*#	30.8 ± 3.3
6 months		
-80°C	32.4 ± 1.7	32.6 ± 2.0
-20°C	26.3 ± 1.3*#	29.9 ± 2.4

* p<0.05 vs. -80°C stored for the same period
\# p<0.05 vs. reference

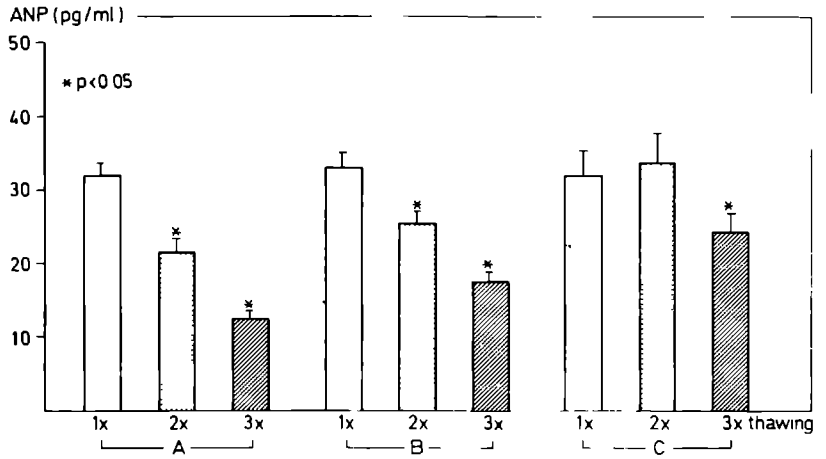

Figure III-5. Influence of repeated thawing of plasma samples from three persons on ANP levels. Bars denote mean ± SD. p = p–value vs. 1 x thawing.

Table III-3. Influence of repeated thawing of plasmas from three subjects. The ANP value of each thawing procedure represents the mean (± SD) of six measurements.

times thawing	subjects		
	A	B	C
1 x	32.2 ± 1.0	33.0 ± 2.0	31.9 ± 3.4
2 x	21.5 ± 1.3*	25.8 ± 1.3*	33.9 ± 3.9
3 x	12.5 ± 1.1*#	17.5 ± 1.3*#	24.7 ± 2.3*#

* p<0.05 vs. 1 x thawing
# p<0.05 vs. 2 x thawing

when kept on ice, for more than one hour decreases ANP immunoreactivity more than 10 %. This figure is higher than that reported by Hartter *et al.* (3). However, these investigators used labelled ANP as standard for measuring the amount of degradation, while we determined the endogenous remaining immunoreactivity, which could explain the difference.

Richards *et al.* (2) reported that platelets interfere with the direct assay for ANP, causing an elevation of ANP concentrations proportional to the number of platelets present in the plasma. Such an effect was not found in our study. However, these blood samples were centrifuged at 1500 g for 5, 10 and 15 minutes, whereas Richards *et al.* centrifuged their samples only at 1000 g for 10 minutes. To analyze whether this could explain the difference, from one person blood was also centrifuged at 1000 g for a 10 minutes period. No significant difference with the other centrifugation methods could be detected (data not shown). These findings imply that in using the methods of measuring ANP as described in this thesis, no special precautions with regard to the amount of platelets are required.

Storage of plasma at −80°C for up to six months did not decrease ANP-immunoreactivity. This is consistent with findings reported by other workers, who did not observe a decrease in ANP levels when plasma was stored at −80°C or even at −20°C (3,4). However, in our study storage at −20°C decreased ANP values markedly in one person. These results imply that plasma should be preferentially stored at −80°C to avoid loss of ANP immunoreactivity. As thawing of plasma markedly decreased ANP-immunoreactivity, samples should be thawed only once before extraction and radioimmunoassay. Other workers have also observed a decrease in ANP-immunoreactivity when plasma was thawed repeatedly (5). To circumvent repeated thawing of plasma when samples have to be assayed more than once, we recommend to divide the plasma after centrifugation over two or more plasma collection tubes.

Considering the results discussed in this chapter, the standard routine for handling blood samples for ANP should be: collection into EDTA-glass tubes on ice, centrifugation within one hour (1500 g, 10 minutes, 4°C), addition of 200 KIU of aprotinin/ml plasma and storage at −80°C. Plasma samples should be thawed only once before assay. All samples used to determine ANP values in this thesis were handled consistently in the above-mentioned method.

Chapter 3.2

Venepuncture stress and menstrual cycle

Introduction

It has been reported that in animal studies stress can elevate ANP levels (6,7). This elevation is probably due to the increase of circulating catecholamine levels (8,9). As venepuncture stress can markedly elevate these levels, venepuncture stress may also influence ANP concentrations.

Progesterone, by antagonizing the binding of aldosterone to its receptor on the distal tubulus of the kidney, acts as a natriuretic agent (10,11). Therefore it cannot be excluded that ANP levels in the follicular phase differ from those in the luteal phase of the menstrual cycle.

These issues were analyzed in the present study.

Methods

To determine if stress caused by venepuncture can influence ANP levels, a catheter was inserted into an antecubital vein of 9 healthy young male subjects who were in the recumbent position for thirty minutes before insertion of the catheter. Blood samples were obtained immediately after insertion of the catheter and 30 minutes thereafter.

For measurement of ANP in two phases of the menstrual cycle, 16 healthy women (aged 18 to 45 years) who were not taking oral contraceptives, were studied on day 4 to 6 (follicular phase) and on day 18 to 19 (luteal phase) of the menstrual cycle. Blood samples were obtained between 9^{00} and 9^{45} a.m. and between 15^{30} and 16^{30} p.m. of the same day.

ANP was determined using the extraction method with the Peninsula antibody. All samples from each person were measured in the same assay run. Statistical analysis was performed using the paired t−test (Student). Significance was assessed at the $\alpha=0.05$ level. Values are expressed as mean ± SD, except where mentioned otherwise.

Results and discussion

In figure III–6 the ANP values immediately after venepuncture (25.4 ± 7.9 pg/ml) and 30 minutes thereafter (29.6 ± 7.1 pg/ml) are shown. No significant decrease was observed between values obtained after 0 and after 30 minutes, suggesting that venepuncture stress does not elevate ANP levels. However, in some persons a disturbing discrepancy was found between the ANP values obtained after 0 minutes and the values obtained 30 minutes later, which cannot be attributed solely to assay variations. As the plasma half life of (99-126)hANP in man is approximately 3.5 minutes (see chapter 4.2), the possibility arises that ANP is not continuously secreted by the heart, but in a pulsatile way.

As shown in figure III–7, ANP levels in the follicular phase tended to be

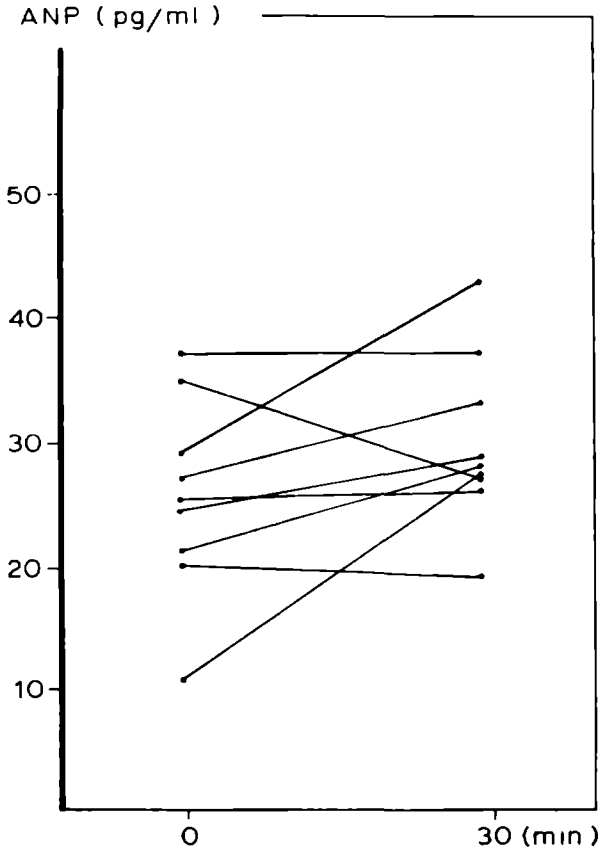

Figure III-6. Influence of venepuncture stress on ANP values in blood sampled at 0 minutes and 30 minutes after insertion of an intravenous catheter. No significant difference was found.

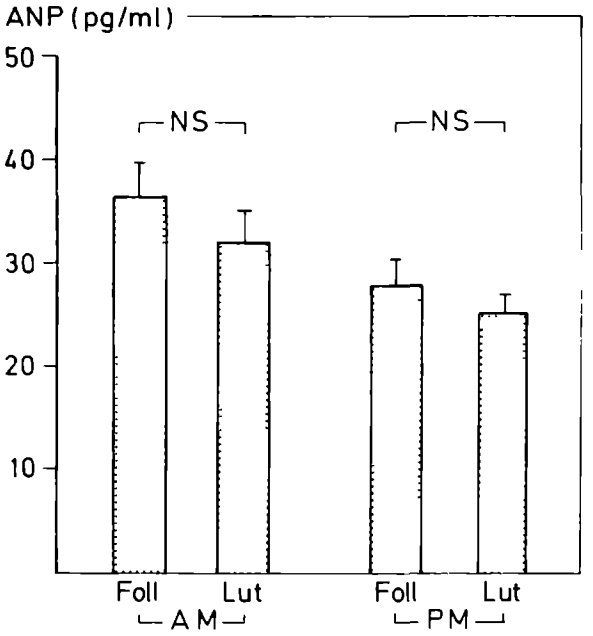

Figure III-7. ANP values (mean ± SEM) in the follicular (foll) and luteal (lut) phase of the menstrual cycle. Blood was sampled in the morning (a.m.) and in the afternoon (p.m.). NS = not significant.

somewhat higher than those in the luteal phase of the menstrual cycle; the difference however was not statistically significant. In contrast, ANP levels obtained in the morning were higher than the corresponding levels obtained in the afternoon (follicular phase: 36.3 ± 13.5 vs. 27.9 ± 11.6 pg/ml; $p<0.05$, luteal phase: 32.1 ± 11.3 vs. 26.9 ± 10.4 pg/ml; $p = 0.06$). Taken the values of both menstrual phases together, the difference between morning and afternoon values was significant (34.2 ± 12.4 vs. 27.4 ± 10.8 pg/ml, $p<0.01$).

In conclusion, both venepuncture stress and the phase of the menstrual cycle do not influence ANP values. A difference however exists between morning and afternoon concentrations of ANP.

Chapter 3.3

Diurnal rhythm

Introduction

As described in chapter 3.2, ANP values obtained in the morning were higher than those obtained in the afternoon. Therefore it could be that ANP, like other hormones, possesses a diurnal rhythm. To explore this possibility, ANP was monitored in 7 ambulant subjects to study the variations during daytime. In addition, ANP was measured three times during a 24 hours period in nine hospitalized patients.

Methods

To study the variation in ANP over the day, an intravenous catheter was inserted into an antecubital vein in five healthy and two pregnant (4 and 8 months) ambulant female volunteers. Blood sampling (from 7^{00} a.m. till 15^{00} p.m., sitting position) was more frequent in the morning. The subjects awoke at approximately 6^{00} a.m., had breakfast at $\pm$ 6^{30} a.m. and lunch at $\pm$ 13^{00} p.m. Statistical analysis was performed using the Spearman rank correlation test on ANP values from each person.

In nine hospitalized patients (7 males, 2 females) without any cardiovascular, renal or endocrine disorders, blood was obtained at 7^{00} a.m., 15^{30} p.m. and at 23^{30} p.m., at least two hours after a meal. The patients were in the supine position during the entire study period. Statistical analysis was performed using analysis of variance for repeated measurements followed by the paired t-test (Student). Significance was assessed at the α=0.05 level. ANP was determined using the extraction method with the Peninsula antibody. All samples from each person were measured in the same assay run.

Results

The variations in ANP levels in a mixed group of ambulant female subjects throughout the day is shown in figure III–8. A statistically significant fall in ANP levels was observed (mean Spearman correlation coefficient $\pm$ SEM = -0.75

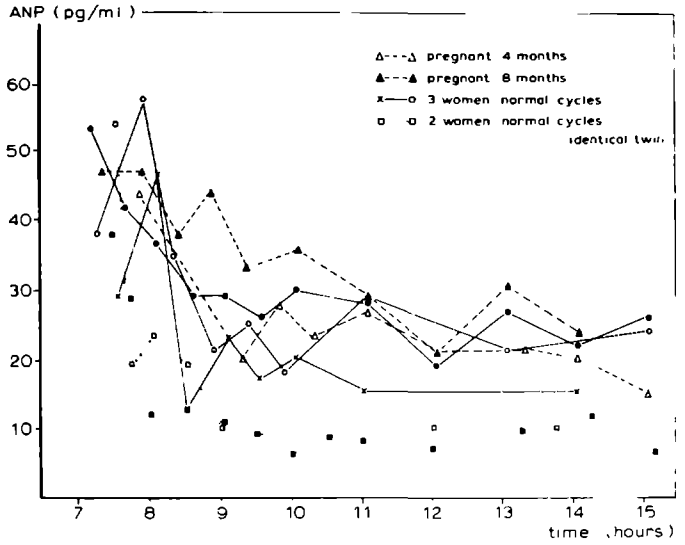

Figure III-8. Variation in ANP levels throughout the day.

± 0.05, $p<0.01$) in all subjects, including the identical twin and the pregnant women. The decline was most prominent between 7^{00} a.m. and 8^{30} a.m., thereafter no significant fall was observed. In the hospitalized patients who were recumbent during the study period, ANP values varied widely intra-individually. In these patients, no consistent trend in ANP concentrations could be detected (figure III–9).

Discussion

The observed fall in ANP concentrations in the early morning in ambulant subjects is in accordance with the results reported by Weil *et al.* These investigators found higher levels of both ANP and cGMP in the morning (12). As a fall in ANP has been described in association with change from the supine to the upright position (see chapter 3.5) and venous pooling of the blood into the lower extremities occurs in the morning (13), this mechanism may be responsible for the observed morning-decline of ANP. The absence of a similar fall in ANP concentrations between 7^{30} a.m. and 15^{30} p.m. in the recumbent patients is in agreement with this hypothesis. Donckier *et al.* (14) reported a nightly peak of ANP at 4^{00} a.m. in the diurnal rhythm of ANP. No variation during daytime was observed in these ambulant subjects. Richards *et al.* (15) found higher ANP

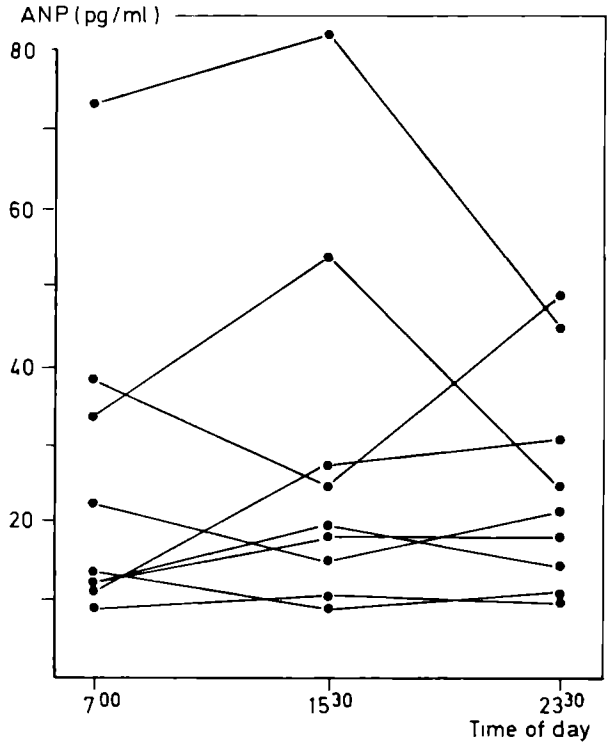

Figure III-9. ANP values sampled in nine patients who were in the supine position during the study period. No consistent pattern in ANP concentrations could be detected.

levels during 11^{00} a.m. and 13^{00} p.m. and lower levels between 17^{00} and 19^{00} p.m. In contrast, other workers (16-18) did not report a diurnal rhythm of ANP. The inconsistent results reported in these studies can be attributed to the frequency and timing of blood sampling, whether ambulant or recumbent subjects were studied, and to the method used to determine ANP concentrations.

In conclusion, ANP values may vary dependent on the time of the day. This factor should be borne in mind when interpreting ANP concentrations.

Chapter 3.4

Intra-individual variation

Introduction

As described in chapters 3.2 and 3.3, a considerable intra-individual variation in ANP levels was observed in some subjects, which could indicate that the heart secretes ANP in a pulsatile way rather than continuously. In order to assess the significance of a possible biological variation in ANP levels, ANP was measured serially during a 52½ minutes period to study the intra-individual variation.

Methods

Fifteen healthy volunteers (8 males, 7 females, aged 20 to 25 years), gave informed consent and, after having fasted overnight, participated in the study. The subjects were not permitted to drink coffee or to smoke cigarettes. After having rested for at least 30 minutes in the supine position, blood (7 ml) was sampled 8 times during a 52½ minutes period (in periods of 7½ minutes each) starting at 9^{00} a.m. ANP was measured using the extraction method with the S−32 antibody. All samples from each person were assayed in the same run. In order to obtain an accurate estimate of the intra-assay coefficient of variation for ANP, the coefficients of variation of 18 different plasma samples (endogenous ANP levels ranging from 12.5 to 55.8 pg/ml, each sample was processed sixfold in the same assay) were determined. The coefficients of variation ranged from 3.0 to 11.4 % (mean ± S.D. = 7.0 ± 2.5 %, median value 6.6 %), and were approximately normally distributed (Shapiro-Wilk normality test $p = 0.6$).

To study the intra-individual variation, the mean and the coefficient of variation (C.V.) of each person were calculated. In order to show the existence of additional variations besides the intra-assay variation, the hypothesis that the intra-individual coefficients of variation belong to the same distribution as the intra-assay coefficients of variation, was tested using the one-sided Wilcoxon two-sample test. Besides, for each subject it was examined whether the subjects's intra-individual coefficient of variation exceeded the intra-assay coefficient of variation using the one-sided t-test (Student). To assess any significant changes in ANP during the study period, differences between the initial and successive ANP values were tested using the paired t-test (Student) with Bonferroni's correction.

Results

ANP levels were measured consecutively eight times at fixed intervals during a 52½ period in 15 healthy volunteers. The mean, standard deviation (SD) and the coefficient of variation (C.V.) were calculated for the data on each individual. The results are depicted in table III–4. The coefficients of variation in these subjects varied widely, from 5.4 % to 23.1 %. Using the one-sided Wilcoxon two-sample test, the hypothesis that the intra-individual coefficients of variation belong to the same distribution as the intra-assay coefficients of variation (7.0 ± 2.5 %) could be rejected at the $\alpha = 0.001$ level. The C.V. in 9 subjects significantly (at the $\alpha = 0.05$ level) exceeded the intra-assay variation, indicating that the intra-individual variation observed in these subjects could not be explained by assay variations alone. During the study period, no consistent

Table III-4. Intra-individual variation of ANP levels in man. The coefficient of variation (C.V.) in 9 out of 15 subjects significantly exceeded the intra-assay variation (7 %) (sex: m = male, f = female, p-value: NS = p>0.10).

Person	sex	mean ANP value (pg/ml, mean ± SD)	C.V. (%)	p-value
A	m	81.7 ± 17.7	21.7	<0.001
B	f	16.5 ± 3.5	21.1	<0.001
C	m	15.4 ± 1.4	8.8	NS
D	f	26.0 ± 4.1	15.7	0.002
E	m	55.5 ± 10.4	18.7	<0.001
F	m	48.1 ± 11.1	23.1	<0.001
G	m	33.2 ± 1.8	5.4	NS
H	f	48.4 ± 3.4	6.9	NS
I	m	23.4 ± 2.9	12.6	0.022
J	f	56.0 ± 3.3	6.3	NS
K	m	33.8 ± 3.8	11.4	0.054
L	m	39.5 ± 7.2	18.2	<0.001
M	f	61.1 ± 8.2	13.5	0.011
N	f	60.1 ± 5.5	9.1	NS
O	f	51.1 ± 6.1	11.9	0.038

change of ANP with time was found.

Discussion

In chapter 3.2, the influence of venepuncture stress on ANP levels was studied. From each person, two blood samples within a period of 30 minutes were collected. Although no consistent change in ANP was observed, some subjects displayed a disturbing discrepancy in ANP values during this 30 minutes period. The study described in this chapter extends these observations. All plasma samples from the same person were assayed in the same run, thereby limiting assay variations. Furthermore, a period of 52½ minutes with blood sampling each 7½ minutes was chosen to limit the possible influence of a diurnal rhythm of ANP. The total amount of blood collected during this period was approximately 60 ml, while haemorrhage of 500 ml did not significantly influence ANP levels (19). The study started at 9^{00} a.m. and the subjects were in the supine position during the entire study period. The possible influence of breakfast was excluded by fasting overnight, while coffee and cigarettes (possibly influencing blood pressure) were not permitted. Despite these precautions, in 9 out of 15 subjects the observed variation in ANP levels could not be attributed to intra-assay variations alone. As no consistent change in ANP levels during this period was observed, the variation cannot be attributed to a diurnal rhythm, the effects of haemorrhage, the effect of assuming the supine position, or to the effect of having had a meal. The only other factor that could possibly explain the observed intra-individual variation, is the existence of endogenous fluctuations in ANP levels. Therefore one should keep this endogenous variation in mind when interpreting ANP concentrations.

Chapter 3.5

Posture and ANP

Introduction

From data obtained during cardiac catheterization in patients with congestive heart failure, it was found that ANP levels are correlated with the right atrial pressure. Increasing the atrial pressure by inflating a balloon in the pulmonary artery concomitantly increases ANP concentrations (20), while decreasing the right atrial pressure by inflating a balloon in the vena cava decreases cardiac ANP secretion (21). In man, the plasma ANP concentration rises after manoeuvres which increase extracellular volume and right atrial pressure (22,23). Furthermore ANP levels have been reported to increase after head-up water immersion, head-down tilt and during ingestion of a high salt diet (24-26). The first two of these manoeuvres are not usually encountered in daily life and the significance of these observations remains uncertain. In particular it is not known whether changes in right atrial pressure, which might occur in normal day to day life, are sufficient to stimulate cardiac ANP secretion. Assuming the upright posture causes venous pooling of blood in the lower parts of the body and hence reduces venous return and right atrial pressure, and this is associated with a reduction in renal blood flow, diuresis and natriuresis, and plasma renin activity (27,28). Inversely, assumption of the supine posture increases central blood volume, venous return, right atrial pressure and renal salt and water excretion.

To investigate the effects of changes in right atrial pressure occurring during daily life, the influence of three posture positions (supine, sitting and upright positions) on ANP secretion was studied. Also, plasma renin activity (PRA) and aldosterone was measured to study the interrelationships between these three volume-regulating hormones.

Methods

A catheter was inserted into an antecubital vein in 19 healthy subjects (12 males, 7 females, aged 20 to 27 years). Blood for measurement of ANP, PRA and aldosterone was withdrawn after one hour in the supine position, 15 minutes later in the sitting position and 15 min thereafter when the subjects were standing upright. Additionally, in 9 of these subjects blood was also sampled 15

minutes later when the subjects were in the supine position again. As the plasma half life of ANP in man is approximately 3.5 minutes (chapter 4.2) a period of 15 minutes was considered to be adequate to detect posture-induced changes in ANP.

ANP was measured using the extraction method with the S−32 antibody. PRA was measured using the Phadebas Angiotensin I test (Pharmacia Diagnostics, Sweden) and plasma aldosterone was measured as described previously (29). All plasma samples from each person were assayed consecutively in the same assay run. Statistical analysis was performed using the Wilcoxon signed rank test and the Spearman rank correlation test. Significance was assessed at the $\alpha=0.05$ level. Values are expressed as mean ± SD, except where mentioned otherwise.

Results

Change of position from supine to sitting significantly decreased ANP levels, while PRA levels did not differ. In contrast, changing from the sitting to the upright position did not further decrease ANP while PRA markedly rose (figure III−10). Plasma aldosterone did not differ in any of the three positions (table III−5). No correlation was observed between the percentual decrease in ANP from the supine to the upright position, and the corresponding percentual increases in PRA ($r = -0.24$, p = NS) or aldosterone ($r = 0.09$, p = NS). Also, the percentual changes in PRA and aldosterone did not correlate ($r = -0.09$, p = NS).

In a subset of nine subjects, hormone levels were also measured after changing from the upright to the supine position again. The results of these subjects are shown in table III−6 and figure III−11. While PRA decreased after changing from the upright to the supine position, no significant change neither in ANP nor in aldosterone was observed. In none of the four positions did ANP values correlate with PRA or aldosterone values. PRA values correlated only in the initial supine position ($r = 0.59$, $n = 19$, $p<0.01$) and in the sitting position ($r = 0.65$, $n = 19$, $p<0.01$) with plasma aldosterone values.

Discussion

This study describes the effects of posture on three hormones influencing the body fluid regulation. ANP can be regarded as a counterregulatory hormone, with effects opposite to those of PRA and aldosterone. In the upright position,

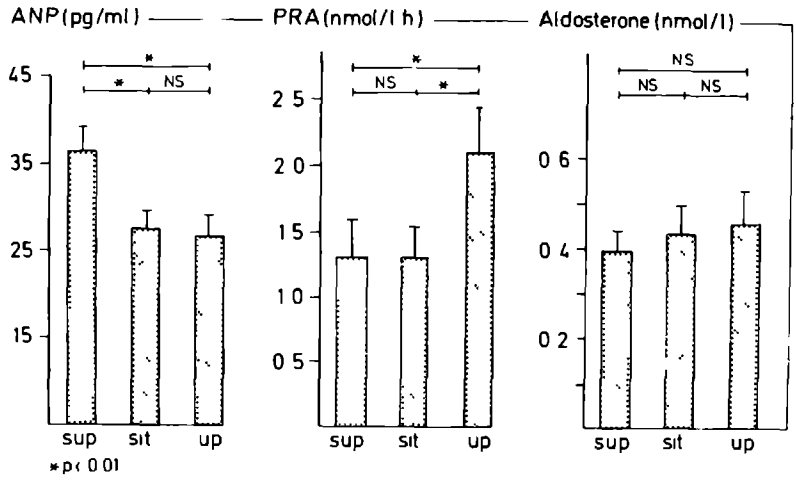

Figure III-10. Influence of posture on plasma concentrations of ANP, PRA and aldosterone (n = 19). Sup = supine, sit = sitting, up = upright position. Bars indicate mean + SEM.

Table III-5. Plasma concentrations of atrial natriuretic peptide (ANP), plasma renin activity (PRA) and aldosterone (Aldo) in the supine, sitting and upright position. Values are expressed as mean ± SD (n = 19).

	Position		
	supine	sitting	upright
ANP (pg/ml)	36.5 ± 14.8	27.5 ± 12.2**	26.7 ± 12.9**
PRA (nmol/l/h)	1.31 ± 1.20##	1.32 ± 1.06##	2.12 ± 1.56**
Aldo (nmol/l)	0.40 ± 0.20	0.44 ± 0.25	0.46 ± 0.32

* p<0.05
** p<0.01
vs. supine position

\# p<0.05
p<0.01
vs. upright position

Wilcoxon signed rank test

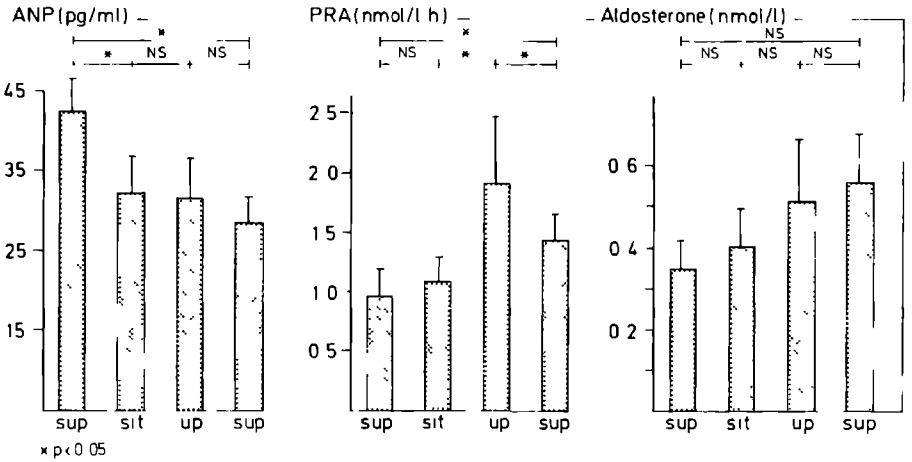

Figure III-11. Position-induced changes in ANP and PRA. Aldosterone levels were not influenced by the position of the subjects (n = 9). Sup = supine, sit = sitting, up = upright position. Bars indicate mean + SEM.

Table III-6. Plasma concentrations of atrial natriuretic peptide (ANP), plasma renin activity (PRA) and aldosterone (Aldo) in 9 subjects, measured in the supine, sitting and upright position, and after assuming the supine position again. Values are expressed as mean ± SD.

	Position			
	supine	sitting	upright	supine
ANP (pg/ml)	42.5 ± 12.5	32.1 ± 13.8*	31.7 ± 15.4*	28.7 ± 8.7*
PRA (nmol/l/h)	0.97 ± 0.61#	1.08 ± 0.65#	1.92 ± 0.96*	1.45 ± 0.71*#
Aldo (nmol/l)	0.35 ± 0.20	0.40 ± 0.30	0.51 ± 0.46	0.56 ± 0.35

* p<0.05 vs initial supine position

\# p<0.05 vs upright position

Wilcoxon signed rank test

lower ANP concentrations were measured as compared to those obtained in the supine position (30,31). Solomon *et al.* (32), using a tilt-table, increased venous return to the heart and observed an increase in ANP concentrations. This increase was markedly attenuated by inflation of antishock-trousers, which apply positive pressure to the legs and lower abdomen thereby diminishing the increase in venous return.

In the study described in this chapter, ANP decreased when the volunteers changed from the supine to the sitting position. However, no further change in ANP was observed when the upright position was assumed. Possibly, more than 15 minutes would be needed for a further decrease in ANP. As infusion of ANP inhibits the postural release of renin (33), an increase in PRA after changing from the supine to the sitting position would be expected. In contrast, PRA increased only when changing from the sitting to the upright position. Assuming the supine position again after standing upright decreased PRA levels while ANP did not change. The lack of correlation between the percentual changes in ANP and PRA is a further indication that during short-term physiological changes the regulation of these two hormones is not reciprocally related.

Chapter 3.6

Exercise and ANP

Introduction

Both in animal studies and in studies performed in humans, ANP levels in peripheral venous blood were found to correlate with intracardiac pressures (5,20). During supraventricular tachycardia and during tachycardia induced by ventricular pacing, elevated ANP levels have been found which returned to baseline after termination of the tachycardia (34,35). Exercise is associated with an increase in heart rate and intra-cardiac pressures (36). Furthermore exercise stimulates adrenaline and noradrenaline levels, which have been found to elevate ANP levels (8,9). To study the influence of exercise on cardiac ANP secretion, a standardized bicycle exercise test was performed on 7 healthy volunteers and its influence on ANP, its second messenger cyclic guanosine monophosphate (cGMP) and various other hormones were investigated.

Methods

The influence of exercise on cardiac ANP secretion was studied on 7 healthy volunteers (4 males, 3 females aged 20 to 28 years). On a separate day prior to the final exercise test, the subjects performed a stepwise graded bicycle exercise test (sitting position) till maximal exhaustion as determined from the expected maximal heart rate for a given age (37). Heart rate was monitored continuously and used to calculate 50 % and 90 % of the maximal workload. On the day of the exercise test, blood was sampled at rest (time = 1), after three minutes of exercise at 50 % of the maximal workload (time = 2), after three minutes of exercise at 90 % of the maximal workload (time = 3) and 15 (time = 4) and 60 (time = 5) minutes after the exercise test. The exercise test started at 14^{00} p.m., ending at 14^{30} p.m.

All samples from each person were measured consecutively in the same assay run. ANP was measured using the extraction method and the S−32 antibody. Cyclic GMP was measured as described in chapter 2.5. Plasma renin activity (PRA) was measured using the Phadebas Angiotensin I test (Pharmacia Diagnostics, Sweden), aldosterone (29), cortisol (38), adrenaline and noradrenaline (39) were measured as described previously. All plasma samples of each person were

measured in the same assay run.

Statistical analysis was performed using the Wilcoxon signed rank test and the Spearman rank correlation test. Significance was assessed at the $\alpha=0.05$ level. Data are expressed as mean ± SD except where indicated otherwise.

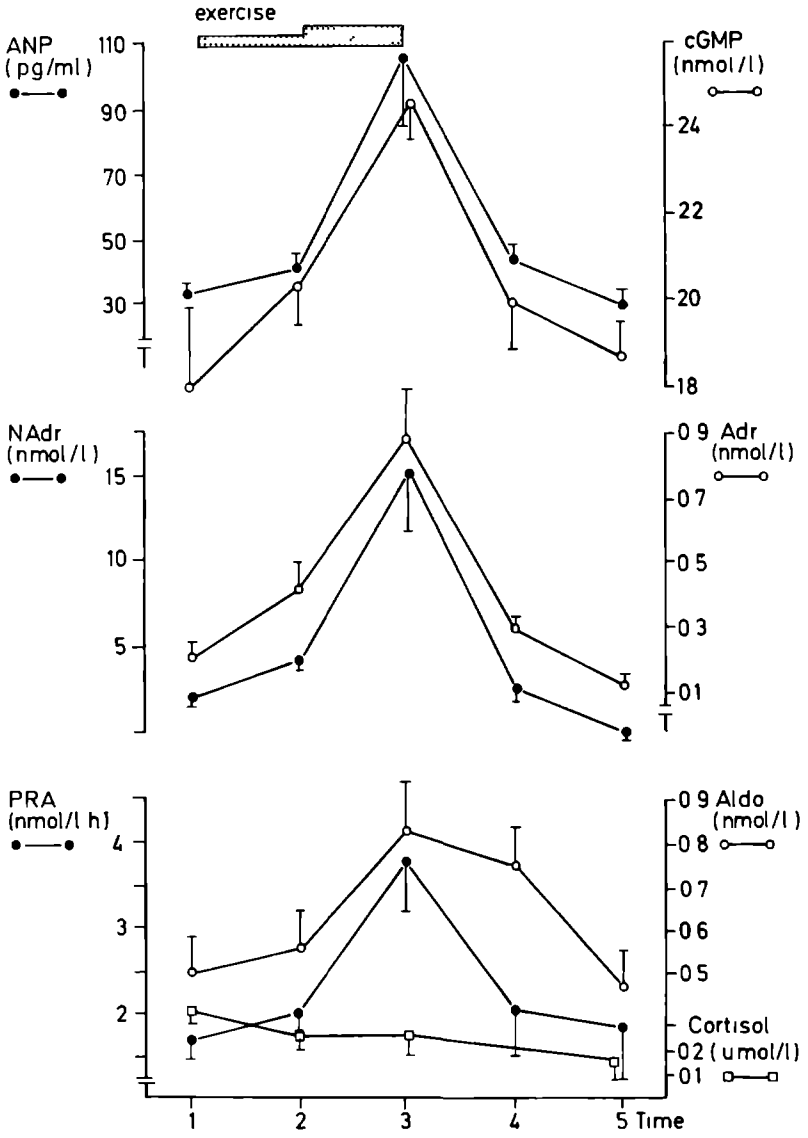

Figure III-12. Influence of exercise on plasma levels of ANP, cGMP, PRA, aldosterone (Aldo), cortisol, adrenaline (Adr) and noradrenaline (NAdr) (n = 7).

Results

Standardized bicycle exercise stimulated both adrenaline and noradrenaline already at 50 % of the maximal effort of the individual subject. PRA and aldosterone increased only at 90 % exercise, while cortisol decreased (figure III–12 and table III–7). ANP levels increased slightly at 50 % exercise, with a marked increase at 90 % exercise. Cyclic GMP values were significantly elevated at 90 % exercise and were positively correlated with the corresponding ANP values ($r = 0.39$, $p<0.05$).

Table III-7. Plasma concentrations of ANP (pg/ml), cyclic guanosine monophosphate (cGMP, nmol/l),noradrenaline (NAdr, nmol/l), adrenaline (Adr, nmol/l), plasma renin activity (PRA, nmol/l/h), aldosterone (Aldo, nmol/l), cortisol (Cor, µmol/l), heart rate (HR, beats/min) and systolic arterial pressure (SAP, mm Hg) at rest (time 1), at 50 % exercise (time 2), at 90 % exercise (time 3) and 15 (time 4) and 60 (time 5) minutes after exercise. Values are expressed as mean ± SD (n = 7).

	Time				
	1	2	3	4	5
ANP	32.6 ±10.3	40.7 ±13.5*#	106.8 ±48.2*	44.0 ±11.7*#	30.7 ±10.8#
cGMP	18.8 ±2.7	20.4 ±2.6#	24.6 ±2.3*	19.9 ±2.65#	18.7 ±2.4#
NAdr	2.21 ±0.55	4.44 ±1.21*#	15.3 ±9.28*	2.59 ±1.15#	1.47 ±0.47*#
Adr	0.22 ±0.12	0.43 ±0.17*#	0.88 ±0.45*	0.30 ±0.11*#	0.13 ±0.08*#
PRA	1.62 ±0.74	1.84 ±0.65#	3.80 ±1.58*	2.06 ±1.17#	1.88 ±1.70#
Aldo	0.50 ±0.20	0.55 ±0.24#	0.83 ±0.29*	0.76 ±0.21*	0.47 ±0.20#
Cor	0.28 ±0.07	0.24 ±0.07*	0.23 ±0.07*	-	0.19 ±0.05*
HR	80.3 ±10.6	34.7 ±9.0*#	182.1 ±8.5*	-	-
SAP	102.7 ±12.6	134.3 ±11.0*#	160.3 ±13.7*	-	-

* $p<0.05$ vs. baseline (time = 1)

\# $p<0.05$ vs. 90 % exercise (time = 3)

Wilcoxon signed rank test

Discussion

The data presented demonstrate that ANP, released during exercise, is capable of exerting biological effects as cGMP values, reflecting the biological effectiveness of ANP (40,41) consistently increase during exercise. In view of the known effects of ANP one would expect a natriuresis and diuresis. During exercise, the opposite phenomenon is observed (42,43). This phenomenon can be attributed to concomitant activation of antinatriuretic and antidiuretic systems such as the sympathetic nervous system and the renin-aldosterone system, which effects override the natriuretic and diuretic effects of the ANP hormonal system. At 90 % of the maximal exercise effort of the individual subject, both PRA and aldosterone rose probably due to activation of the sympathetic nervous system as shown by the increase in adrenaline and noradrenaline.

Rather unexpectedly (44), plasma cortisol decreased during exercise. Odink *et al.* (45) and Cumming *et al.* (46) also found no change or a slight decrease in cortisol values during exercise, rising shortly after exercise. As no cortisol values were measured shortly after exercise, their observations could not be confirmed.

In patients with ischaemic heart disease, a disproportionate elevation of ANP during exercise was found as compared to patients without ischaemic heart disease (47,48). This may be due to a disproportionate elevation of intracardiac pressures during exercise in the former group. Furthermore ANP levels were correlated to maximal creatine kinase levels and were inversely correlated to the left ventricular ejection fraction (47) and to the maximal oxygen uptake, which uptake is an index for the remaining ventricular function (49).

In conclusion, levels of both ANP and its second messenger cGMP consistently increase during a standardized bicycle exercise test in healthy volunteers. This test may be useful as a noninvasive method for evaluating cardiac function.

Chapter 3.7
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Part IV

AGE–DEPENDENCY OF ANP

In elderly subjects higher plasma ANP concentrations have been reported as compared to those found in young subjects (1,2). One explanation for these higher levels in elderly subjects could be a difference in clearance of ANP. Therefore synthetic (99-126)hANP was infused in both young and elderly subjects and the pharmacokinetic parameters were compared. The physiological significance of the higher ANP concentrations in elderly subjects is not yet known. Two possibilities arise: the sensors governing ANP release by the heart are more sensitive in elderly subjects, or a down-regulation of ANP target-organ responsiveness occurs in these subjects with a compensatory increase in ANP secretion. As the secretion of ANP is dependent on the intravascular volume, the influences of dehydration (causing a slight volume contraction) and volume loading (causing volume expansion) on ANP concentrations in young and elderly subjects were studied. Furthermore, in order to assess directly the influence of age on haemodynamic and endocrine effects of ANP, (99-126)hANP was infused both in young and in elderly subjects and the effects in both groups were compared. Cyclic guanosine monophosphate (cGMP) functions as the second messenger for ANP. As cGMP levels can be measured in plasma, the comparison between young and elderly subjects was extended by using cGMP levels as an index of the biological effectiveness of ANP.

Chapter 4.1

Age and immunoreactive ANP concentrations

Introduction

Various investigators have observed higher basal concentrations of immunoreactive ANP in elderly subjects as compared to young subjects (1,2). However, in these studies ANP concentrations found in one group of elderly subjects were compared with those found in one group of young subjects.

In order to obtain normal values for ANP, blood was sampled from 66 healthy subjects varying in age from 22 to 79 years. Within this study group a linear correlation was found between the age of the person and the corresponding ANP concentration. As this finding confirms the assumption that immunoreactive ANP concentrations increase with advancing age and the ANP immunoreactive profile in healthy persons is heterogeneous (chapter 2.4), one may wonder whether the higher ANP concentrations in elderly subjects can be attributed to different circulating forms of ANP as compared to young subjects. Therefore the ANP immunoreactive profiles of one young and one elderly person were studied, both in the basal state and after stimulation by volume loading.

Methods

To study the influence of age on plasma levels of ANP, blood was obtained from 66 healthy persons on free sodium intake, without a history of cardiovascular, renal or endocrine disorders, between 9^{30} and 10^{00} a.m. after 30 minutes in the sitting position and after at least 3 hours of fasting. None of the volunteers used any medications, oral anticonceptives included. The age of the volunteers (29 females, 37 males) varied from 22 to 79 years. Care was taken that the sex and number of volunteers was evenly distributed over all age classes. Systolic arterial pressure (SAP), diastolic arterial pressure (DAP) and heart rate were recorded. Mean arterial pressure (MAP) was calculated as MAP = DAP + (SAP - DAP)/3. ANP was measured using the extraction method and the S−32 antibody. Correlations were made using the Spearman rank correlation test. Significance was assessed at the $\alpha=0.05$ level. Blood from one young and one elderly person (both basal and after stimulation by volume loading, see chapter 4.4) for HPLC analysis was collected into EDTA tubes on ice and centrifuged

immediately. HPLC analysis was performed as described in chapter 2.4.

Results

The values obtained from 66 healthy volunteers ranged from 8.3 to 86.8 pg/ml (mean ± SD = 35.3 ± 16.2 pg/ml). Although a wide range of ANP values was observed, the ANP concentration correlated positively with the subject's age (Spearman r = 0.57, $p<0.01$, figure IV−1). Both mean arterial pressure and systolic arterial pressure correlated positively with the ANP concentration (r = 0.27, $p<0.05$ and r = 0.28, $p<0.05$, respectively). However, these correlations disappeared when a correction for age was applied. Neither heart rate nor diastolic arterial pressure correlated with the ANP value.

In figure IV-2 the HPLC profiles of one young and one aged person are shown. The HPLC profile of the aged person was similar to that of the young person. Both in the basal state and after stimulation the predominant immunoreactive form corresponds to (99-126)hANP.

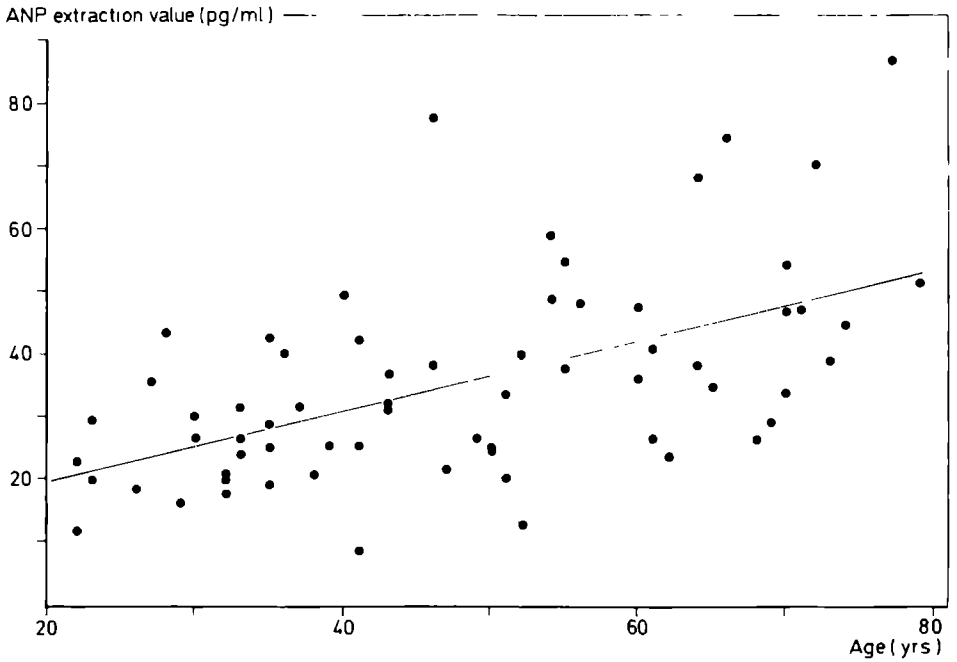

Figure IV-1. ANP values in 66 healthy subjects ranged from 8.3 to 86.8 pg/ml (mean ± SD = 35.3 ± 16.2 pg/ml). ANP values correlated significantly with age (r = 0.57, p<0.01).

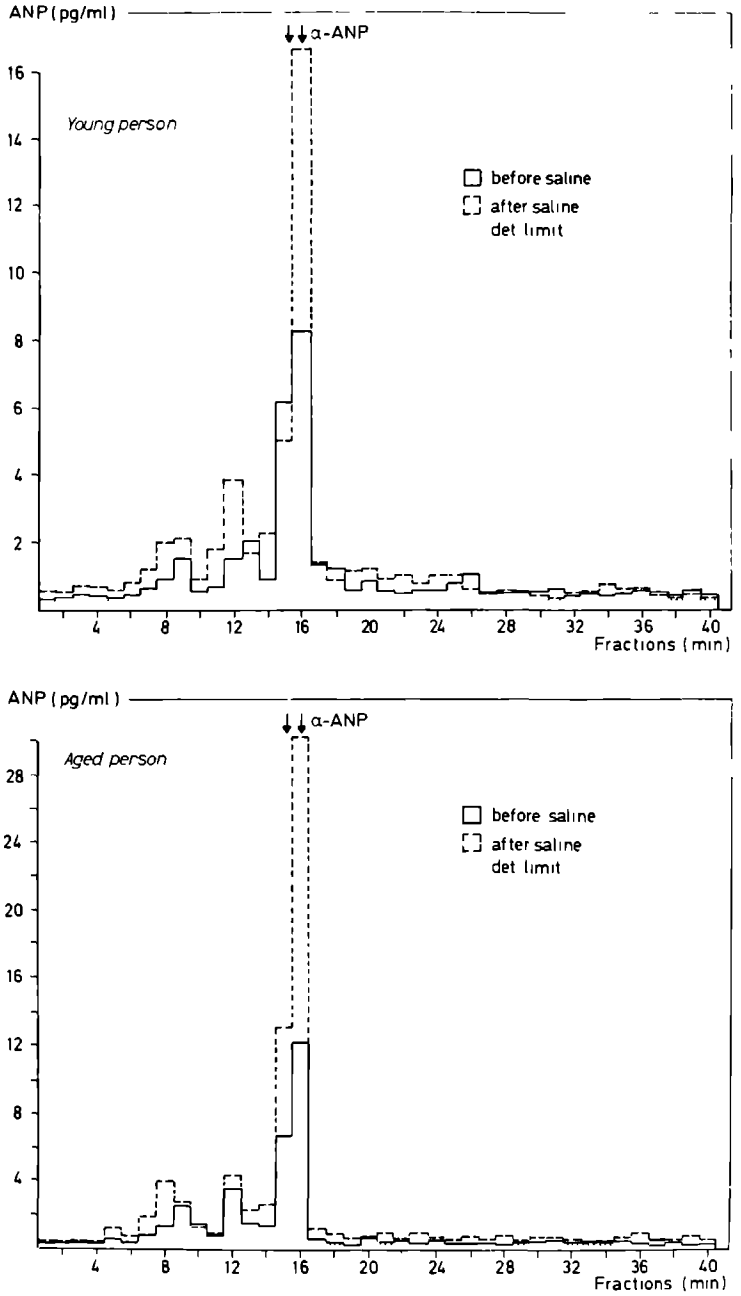

Figure IV-2. HPLC-profile of immunoreactive ANP before and after volume loading in one young person (upper panel) and one aged person (lower panel). The main immunoreactive component corresponds to (99-126)hANP (= α-ANP).

Discussion

ANP concentrations as assessed in blood from 66 healthy subjects ranged from 8 to 87 pg/ml. Blood was obtained under standardized conditions. To minimize external factors that might influence the endogenous ANP level, blood was sampled in the morning between 9^{30} a.m. and 10^{00} a.m., after half an hour in the sitting position. Furthermore, care was taken that both sexes and all age classes were evenly represented. As these subjects were recruited from the general population, this wide range of ANP values can be regarded as a measure of ANP concentrations found in the normal population. Within this study group, a significant correlation between the ANP value and the age of the subject was observed, with plasma levels in elderly subjects (70 years and older) being almost twice that of younger subjects (20 to 25 years). These results indicate that ANP concentrations increase progressively with advancing age and that the higher ANP levels observed in elderly subjects do not represent alterations in ANP production/metabolism present only in this age group. The generally higher ANP levels in elderly subjects may not be attributable to different forms of ANP released by the heart as the HPLC profile of the aged person was similar to that of the young person. The predominance of the (99-126)hANP form both before and during stimulation is in agreement with the hypothesis that this molecular form of ANP is the most important circulating form of ANP in humans (3,4).

Chapter 4.2

Clearance of ANP

Introduction

As described in chapter 4.1, ANP concentrations in healthy subjects increase with advancing age. One explanation for these higher levels could be a diminished clearance of ANP in elderly subjects as compared to young subjects. To investigate this possibility the pharmacokinetic parameters of ANP were determined in 8 healthy young and in 9 healthy elderly subjects.

Methods and study design

Study design

The study protocol was approved by the local Hospital Ethical Committee. After giving informed consent, 8 healthy young (aged 18 to 25 years, 4 males and 4 females) and 9 healthy old (aged 71 to 84 years, 4 males and 5 females) subjects not using any medications, participated in the study. Prior to the study a physical examination was performed to exclude any existing cardiovascular, renal or endocrine disorders. (99-126)hANP was purchased from Bissendorf Peptide GmbH, Wedermark, FRG. After a 60 minutes equilibration period in the supine position, ANP (dissolved in Haemaccel) was infused into an antecubital vein for 60 minutes at a rate of 2.0 μg/min. Blood for determination of ANP was sampled 30 minutes and just before infusion, at 15, 30, 45 and 60 minutes of infusion, and 1, 3, 5, 7, 10, 15, 20, 30, 40 and 60 minutes after termination of infusion.

Methods

ANP was measured using the extraction method and the S-32 antibody. Assuming that elimination of ANP is a first-order process, the plasma half-life ($t_{½}$) was calculated using linear regression analysis on logarithmic ANP values after termination of the infusion. The total body clearance (Cl_{TB}) was calculated dividing the dose by the area under the curve (determined from the ANP concentration at t = 0 until the ANP concentration at t = 120 minutes using a linear trapezoidal method). The volume of distribution (V_d) was calculated by dividing the Cl_{TB} by the elimination constant K_{el} (calculated as $\ln 2/t_{½}$). The endogenous

creatinine clearance (ECC) was calculated from the serum creatinine level (5) and normalized for the body surface area (6).

Statistical analysis

The pharmacokinetic parameters of the young and the elderly subjects were compared using the Wilcoxon two-sample test and the Spearman rank correlation test. Significance was assessed at the $\alpha=0.05$ level. All values are expressed as mean ± SD, except where mentioned otherwise.

Results

As shown in table IV−1, no differences in height, weight or quetelet-index (QI) were found between the young and the elderly subjects. The endogenous

Table IV-1. Pharmacokinetic parameters of young v.s. elderly subjects. Values are expressed as mean ± SEM. For abbreviations see text. P - value between groups:
* $p<0.05$ ** $p<0.01$ NS = non-significant.

	young n=8	p	elderly n=9
Age (years)	23.3 ± 1.0		75.3 ± 1.4
Height (cm)	175.0 ± 3.1	NS	163.1 ± 4.6
Weight (kg)	68.9 ± 5.5	NS	66.3 ± 4.0
QI (kg/m^2)	22.4 ± 1.5	NS	24.9 ± 1.2
ECC ($ml/min/1.73\ m^2$)	109.6 ± 5.2	**	49.7 ± 3.7
ANP_{bas} (pg/ml)	33.8 ± 4.0	*	51.3 ± 4.1
ANP_{inf} (pg/ml)	562 ± 89	**	1055 ± 130
$t_{½}$ (min)	3.72 ± 0.34	NS	3.52 ± 0.37
Cl_{TB} (l/min)	6.02 ± 1.45	*	2.86 ± 0.33
V_d (liter)	32.8 ± 9.0	*	14.1 ± 1.9

creatinine clearance (ECC), normalized for the body surface area, was markedly lower in the elderly subjects (values ranging from 31.5 to 64.1 ml/min/1.73 m^2) as compared to the young subjects (values ranging from 78.7 to 125.5 ml/min/1.73 m^2). Both baseline ANP values (ANP_{bas}, mean of the values obtained 30 minutes before and just prior to the start of the infusion) and the values at the end of the infusion (ANP_{inf}) were significantly higher in the elderly (figure IV–3). The plasma half-life (t½) of ANP was similar in both groups (young: range 1.89 to 4.57 minutes, aged· range 1.96 to 5.18 minutes, p = NS). In contrast, the total body clearance (Cl_{TB}, young: range 3.11 to 14 36 l/min, aged: range 1.09 to 4.36 l/min, $p<0\,05$) and the volume of distribution (V_d, young: range 8.5 to 89.4 liter, aged: range 5.2 to 24.9 liter, $p<0.05$) were markedly diminished in the elderly subjects (table IV–1)

Within the elderly, but not within the young subjects a positive correlation (young: r = 0 50, p = NS, aged. r = 0.95, $p<0\,01$) was observed between the ECC and the Cl_{TB} (figure IV–4). Basal ANP values were not related to the ECC, neither within the young (r = 0.43, p = NS) nor within the elderly (r = −0.48, p = NS) subjects.

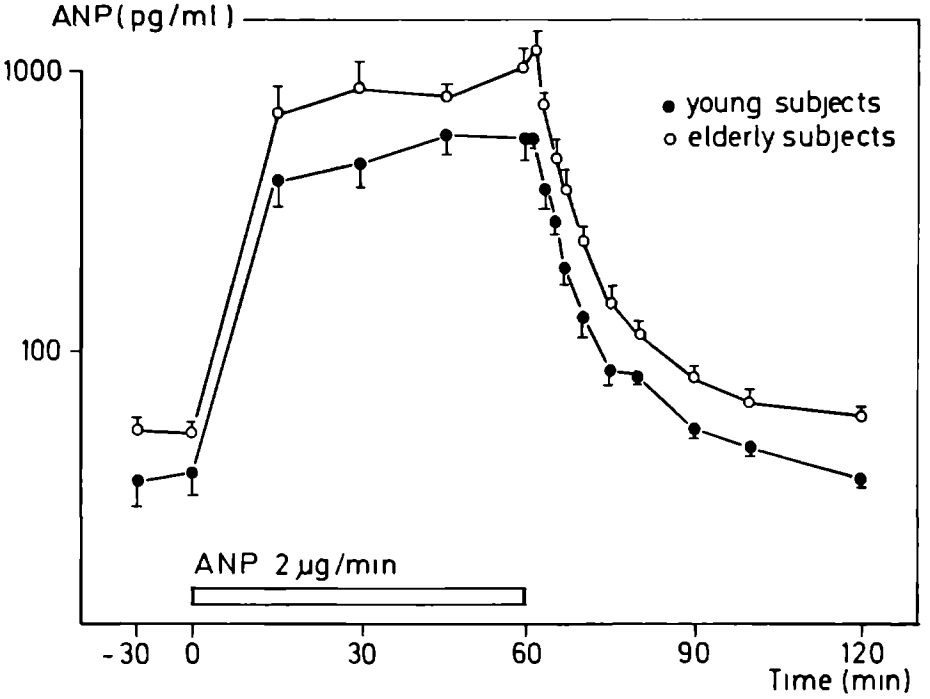

Figure IV-3. ANP values (log scale) before, during and after infusion of (99-126)hANP (2.0 µg/min) in young and elderly subjects. Values are mean ± SEM.

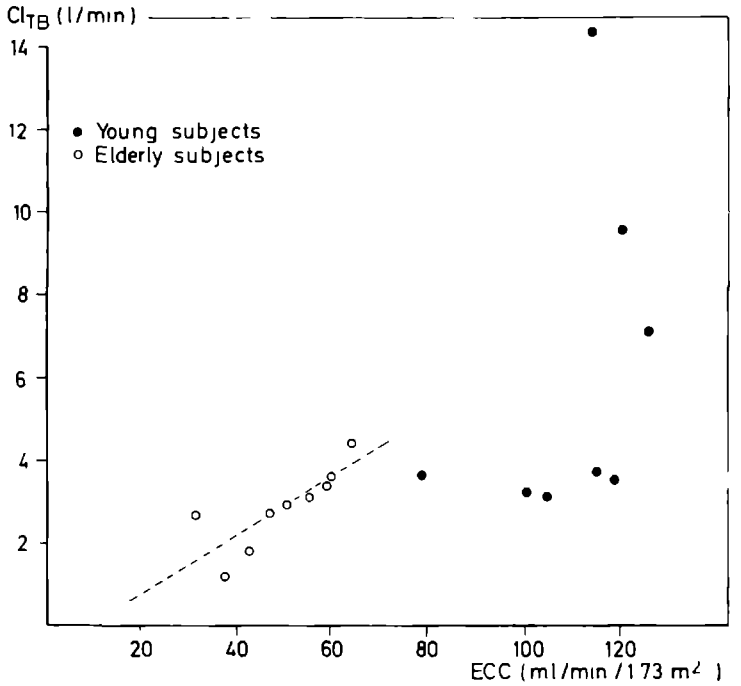

Figure IV-4. Relationship between the total body clearance of ANP (Cl_{TB}) and the endogenous creatinine clearance (ECC), representing glomerular filtration rate, in young (r = 0.50, p = NS) and elderly (r = 0.95, p<0.01) subjects.

Discussion

Using a one-compartment model and first-order pharmacokinetics, a similar t½ but a markedly diminished Cl_{TB} and consequently a diminished V_d was observed in the aged persons. This decreased V_d could explain the higher ANP levels generally found in elderly subjects. The t½, Cl_{TB} and V_d found in this study are well in line with those found in other studies (7-9). Ohashi *et al.* (10), using bolus injections of ANP, observed a prolonged second phase of (99-126)hANP disappearance and a decreased metabolic clearance rate in elderly subjects. These investigators used a biexponential equation for their calculations. As ANP is an endogenously secreted hormone, the assumption of a biexponential disappearance curve may not be entirely correct. Furthermore in their study ANP was measured using a direct assay. The direct assay is subject to interference from plasma components, giving less reliable results than an assay based on prior extraction of

plasma (11,12).

As ANP is degraded by the kidney (13-16) and not excreted in the urine, the Cl_{TB} probably represents metabolic clearance rather than renal clearance of ANP. Marumo *et al.* (17) using a direct assay, detected immunoreactive ANP in human urine. This immunoreactivity is probably caused by the presence of urodilatin, a 32 amino acids containing polypeptide corresponding to (95-126)ANP which is produced locally in the kidney and secreted into the urine (18).

In man, the lung, liver and kidney contribute substantially to the metabolic removal of ANP from the circulation (19). Luft *et al.* (20) showed that the t½ of ANP was markedly prolonged in anephric rats as compared to control rats. Hollister *et al.* (21) observed in hypertensive humans a high ANP extraction ratio of the kidney but, due to the lower blood flow, a lower clearance rate of ANP as compared to the lung and the liver. Also, a positive correlation was found between endogenous creatinine clearance and ANP clearance. More than 95 % of ANP receptors in rat kidney are biologically silent (22). Binding of ANP to these receptors (called C-ANP receptors) does not result in a functional response or in cGMP production, in contrast to the B-ANP receptors mediating the biological effects of ANP through its second messenger cGMP. When these C-ANP receptors are specifically blocked by ring-deleted ANP analogues the endogenous plasma ANP concentrations increase thereby suggesting that these C-ANP receptors possess an important ANP clearance function (22).

In elderly subjects the endogenous creatinine clearance (ECC), an estimate of the glomerular filtration rate, corresponds to the number of functioning nephrons (23). The excellent correlation observed between the Cl_{TB} and the ECC in these subjects therefore supports the assumption that the kidney is a major determinant of ANP clearance.

Chapter 4.3

Dehydration and ANP

Introduction

The primary stimulus for secretion of atrial natriuretic peptide (ANP) by the heart is stretch of cardiac myocytes (24,25). Immunoreactive ANP levels in human blood rise after volume expansion induced by saline infusion (26,27). Even small changes in central blood volume caused by water immersion, head-down tilt or during ingestion of a high salt diet are able to stimulate cardiac ANP secretion (28-31).

Dehydration is associated with contraction of the body fluid volume. One would therefore expect a decrease in ANP levels. As in particular elderly subjects are prone to dehydration the effect of mild dehydration by restricting fluid intake to 25 % of normal on plasma levels of ANP and antidiuretic hormone (ADH) in relation to renal concentrating capacity was studied in young as well as elderly subjects.

Methods and study design

Dehydration study

Thirteen young (8 males, 5 females, mean age ± SD = 21.8 ± 1.8 years, range 18 to 25 years) and 10 elderly (4 males, 6 females, mean age ± SD = 76.3 ± 4.1 years, range 72 to 86 years) subjects without renal, cardiovascular or endocrine disorders, gave informed consent and participated in the study. On two separate days preceding the study, the fluid intake of each subject was measured and noted by diary. On the day prior to the study, a 24 hour urinary collection was made for measurement of urinary osmolality, free water clearance and osmolar clearance. Thereafter the subjects were restricted to 25 % of their normal fluid intake for one day, and a 24 hour urinary collection was made. Both before and after the study, blood was sampled for determination of plasma osmolality, haematocrit, ANP, and antidiuretic hormone (ADH).

Hormonal and other measurements

ANP was measured using the extraction method and the S−32 antibody. After prior precipitation of plasma proteins with ethanol, ADH levels were measured

using the Vasopressin-Rapid assay from Bühlmann Laboratories AG, Switzerland. Osmolar clearance (Cl_{osm}) was calculated as $Cl_{osm} = U_{osm} \times V / P_{osm}$ (U_{osm} = urinary osmolality, V = urinary volume, P_{osm} = plasma osmolality). Free water clearance (Cl_{H2O}) was calculated as $Cl_{H2O} = V - C_{osm}$.

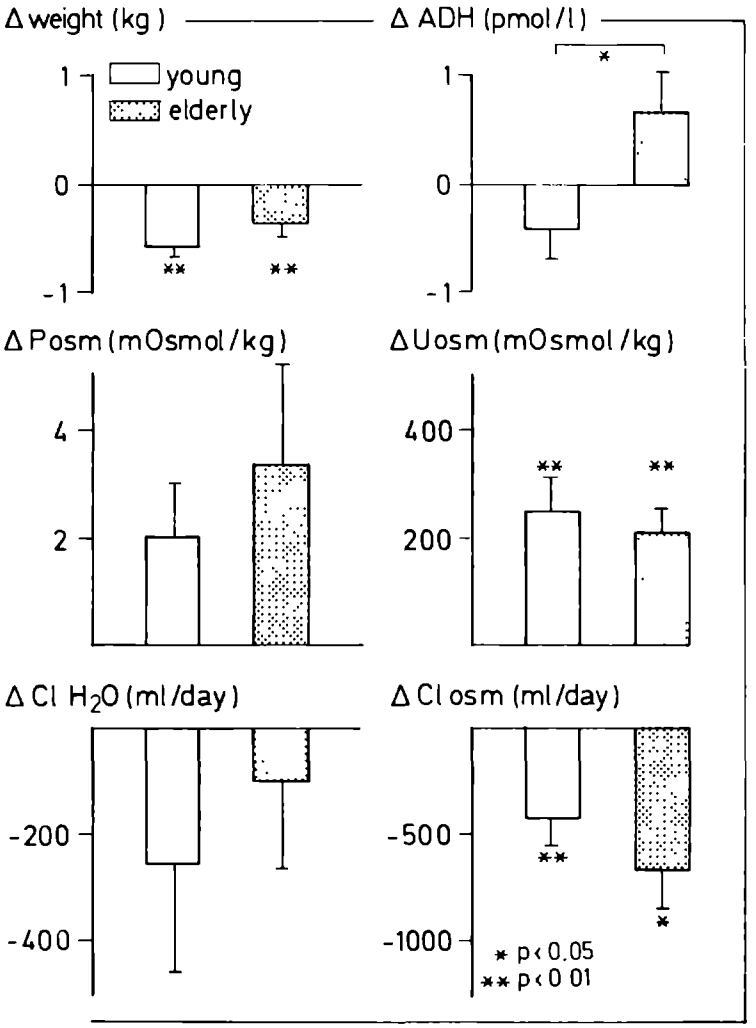

Figure IV-5. Changes in weight, antidiuretic hormone (ADH), plasma osmolality (P_{osm}), urinary osmolality (U_{osm}), free water clearance (Cl_{H2O}) and osmolar clearance (Cl_{osm}) during the dehydration study in young and elderly subjects. Values are mean ± SEM.

Statistical analysis

Differences before and after dehydration were compared using the Wilcoxon two-sample test and the Wilcoxon signed rank test. Significance was assessed at the $\alpha=0.05$ level. All values are expressed as mean ± SD except where mentioned otherwise.

Results

Restriction of fluid intake to 25 % of normal significantly increased urinary osmolality (U_{osm}) in both the young and the aged persons, while body weight decreased. Free water clearance (Cl_{H2O}) decreased in both groups non-significantly and osmolar clearance (Cl_{osm}) decreased significantly (figure IV−5). Plasma antidiuretic hormone (ADH) levels tended to increase in the elderly and tended to decrease in the young. The difference between the young and the elderly groups was significant. ANP levels did not show a consistent change during the dehydration study neither in the young nor in the elderly subjects (figure IV−6). Both before and after the study, ANP levels were significantly higher in the elderly persons as compared to the younger persons (table IV−2). In contrast, basal ADH levels were higher in the young persons.

Discussion

Restriction of fluid intake to 25 % of normal increased plasma and urinary osmolality and decreased free water clearance in both groups. Also, a relative underfilling of the cardiovascular compartment occurred, as shown by the decrease in osmolar clearance. These results are in agreement with other reports on the influence of dehydration on the water and salt homeostasis (32,33). Interestingly, ADH showed a tendency to increase in the elderly and to decrease in the young subjects, while the ability of the kidney to retain salt and water was similar in both groups. This may point to decreased responsiveness of the kidney to ADH with advancing age (32). In hyperosmolar volume states, neither the ADH concentration nor the plasma osmolality but rather the volume of the intravascular compartment determines cardiac ANP release (34,35). In view of the volume contraction during dehydration, one would expect ANP levels to decrease. However, neither in the young nor in the elderly subjects a consistent change of ANP occurred during the dehydration study. Within the elderly subjects ANP values even tended to rise. Several investigators showed that acute

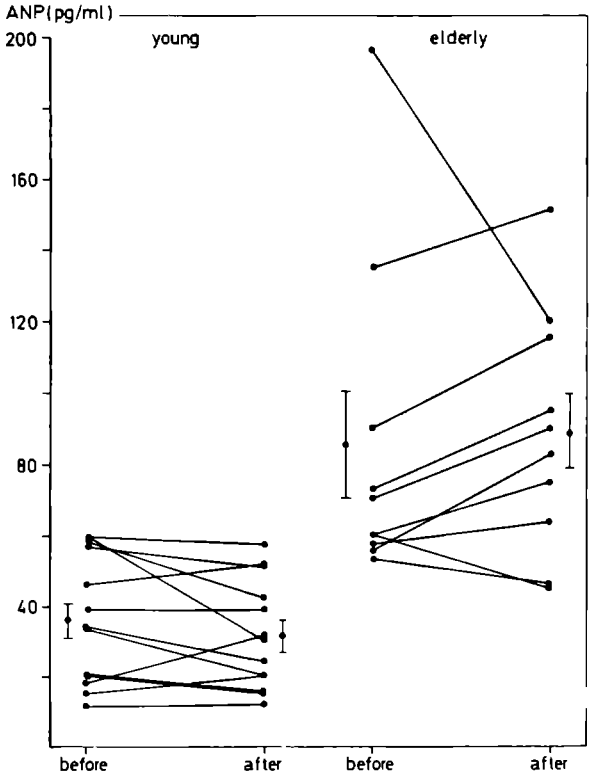

Figure IV-6. Individual changes (and mean ± SEM) in ANP values during the dehydration study. Neither in the young nor in the elderly subjects a significant change was observed.

volume depletion induced by furosemide administration lowers ANP levels (36,37). The absence of a decrease in ANP levels in our study may be explained by the following. It is possible that the dehydration induced by restriction of fluid intake to 25 % of normal did not cause sufficient volume contraction or that despite the volume contraction, counterregulatory mechanisms prevented a decrease in central (or so-called "effective") blood volume which is the main determinant for release of ANP. In either case, these results show that mild dehydration does not affect ANP concentrations in humans.

Table IV-2. Values before and after dehydration (mean ± SD), the difference and the p-value for differences between groups (young vs. elderly). For abbreviations see text (P_{osm} = plasma osmolality, Ht = haematocrit). * $p<0.05$ ** $p<0.01$ vs. elderly persons.

Young persons

	Before	After	Difference	p
Weight (kg)	68.5 ± 5.4	67.9 ± 5.5	-0.58 ± 0.28	p<0.01
P_{osm} (mosm/kg)	285.5 ± 3.8	287.5 ± 4.8	2.00 ± 3.67	NS
U_{osm} (mosm/kg)	637.2 ± 259.7	890.0 ± 170.2	253 ± 247	p<0.01
Cl_{H_2O} (ml/day)	-1538 ± 937	-1797 ± 411	-259 ± 700	NS
Cl_{osm} (ml/day)	3123 ± 623	2705 ± 475	-418 ± 447	p<0.01
Ht (l/l)	0.40 ± 0.03	0.40 ± 0.03	0.004 ± 0.02	NS
ADH (pmol/l)	7.70 ± 1.83*	6.88 ± 1.00	-0.90 ± 1.6*	NS
ANP (pg/ml)	36.5 ± 18.2**	31.9 ± 15.5	-4.6 ± 10.8	NS

Elderly persons

	Before	After	Difference	p
Weight (kg)	74.9 ± 8.6	74.2 ± 8.6	-0.39 ± 0.28	p<0.01
P_{osm} (mosm/kg)	287.8 ± 3.1	291.2 ± 4.5	3.40 ± 5.85	NS
U_{osm} (mosm/kg)	494.3 ± 135.7	710.9 ± 115.1	216.6 ± 142.7	p<0.01
Cl_{H_2O} (ml/day)	-1083 ± 541	-1183 ± 323	-100.3 ± 530.8	NS
Cl_{osm} (ml/day)	2699 ± 655	2031 ± 469	-668 ± 548	p<0.05
Ht (l/l)	0.38 ± 0.04	0.38 ± 0.03	-0.006 ± 0.009	NS
ADH (pmol/l)	5.91 ± 0.97	7.30 ± 2.45	1.39 ± 2.29	NS
ANP (pg/ml)	86.0 ± 46.2	89.3 ± 34.0	3.31 ± 31.4	NS

Chapter 4.4

Volume loading and ANP

Introduction

In healthy subjects a positive correlation between the ANP concentration and the age of the subject was observed (chapter 4.1). One possible explanation for the higher ANP concentrations found in elderly subjects could be an increased sensitivity of the volume regulating sensors governing ANP release. As the secretion of ANP is dependent on the circulatory volume of the intravascular compartment the influence of volume expansion (750 ml 0.9 % NaCl in 60 minutes) on ANP levels in young as well as elderly subjects was studied. Cyclic guanosine monophosphate (cGMP) functions as the second messenger for the ANP hormonal system (38,39). As cGMP concentrations can be measured in human plasma, determination of cGMP levels roughly reflects the biological effectiveness of ANP (40,41).

Methods and study design

The study protocol was approved by the local Hospital Ethical Committee. All subjects gave informed consent and underwent a thorough clinical examination (including electrocardiography and a chest X-ray) to exclude renal, cardiovascular or endocrine disorders. None of the subjects used any medications and refrained from alcohol, coffee and cigarettes for 24 hours preceding the study.

Volume loading

Eight young (4 males, 4 females, mean age ± SD = 22.5 ± 0.8 years, range 21 to 23 years) and 8 elderly (4 males, 4 females, mean age ± SD = 73.1 ± 5.5 years, range 65 to 84 years) subjects participated in the study. The study started at 8^{30} a.m. After having fasted overnight, the subjects rested for one hour in the recumbent position. Thereafter 750 ml 0.9 % NaCl was infused into an antecubital vein during one hour. Blood for determination of ANP, cGMP, plasma renin activity (PRA) and aldosterone was sampled 15 minutes before and just prior to the saline infusion, at 30, 45 and 60 minutes of infusion, and 30, 60 and 90 minutes after termination of the infusion. Plasma volume expansion was calculated according to the formula $[TP_1 - TP_2]/TP_1 \times 100\%$ where TP_1 is the total

protein content in plasma before and TP_2 is the total protein content in plasma after saline infusion. The percentual change in ANP values was calculated according to the formula $[ANP_{max} - ANP_{bas}]/ANP_{bas} \times 100\%$ where ANP_{max} is the maximal ANP concentration during the study period and ANP_{bas} is the mean of the ANP values obtained 15 minutes before and just prior to the saline infusion. A similar formula was used to calculate the percentual change in cGMP values.

Hormonal measurements

ANP was measured using the extraction method and the S-32 antibody. Cyclic GMP was measured as described in chapter 2.5. PRA was measured using the Phadebas Angiotensin I test (Pharmacia Diagnostics, Sweden), plasma aldosterone was measured as described previously (42).

Statistical analysis

The responses of the various hormones to saline infusion were studied using analysis of variance for repeated measurements followed by the paired t-test (Student). Differences in plasma volume expansion were analyzed using the Wilcoxon two-sample test and correlations were made using the Spearman rank correlation test. To assess differences in rise time to the maximum ANP and cGMP values, the Wilcoxon two-sample test and the Wilcoxon signed rank test were used. Significance was assessed at the $\alpha=0.05$ level. All values are expressed as mean ± SD except where mentioned otherwise.

Results

Basal ANP values were higher in the elderly persons. Volume loading increased ANP only in the elderly subjects, while no significant change occurred in the younger persons. PRA decreased only in the younger subjects while plasma aldosterone did not change in either group (figure IV−7 and table IV−3). Basal cGMP levels did not differ between the young and the aged persons. During volume loading, cGMP increased in both groups with a more pronounced rise in the elderly subjects. The difference between the two groups however, was not statistically significant (two-way repeated measurements analysis of variance). No correlation was observed between basal cGMP and ANP values (young: $r = -0.09$, $p = NS$, aged: $r = 0.33$, $p = NS$), nor between the percentual change in cGMP and the percentual change in ANP values in either group (young: $r = 0.52$, $p = NS$, aged: $r = 0.01$, $p = NS$).

During the saline infusion study, a significant plasma volume expansion

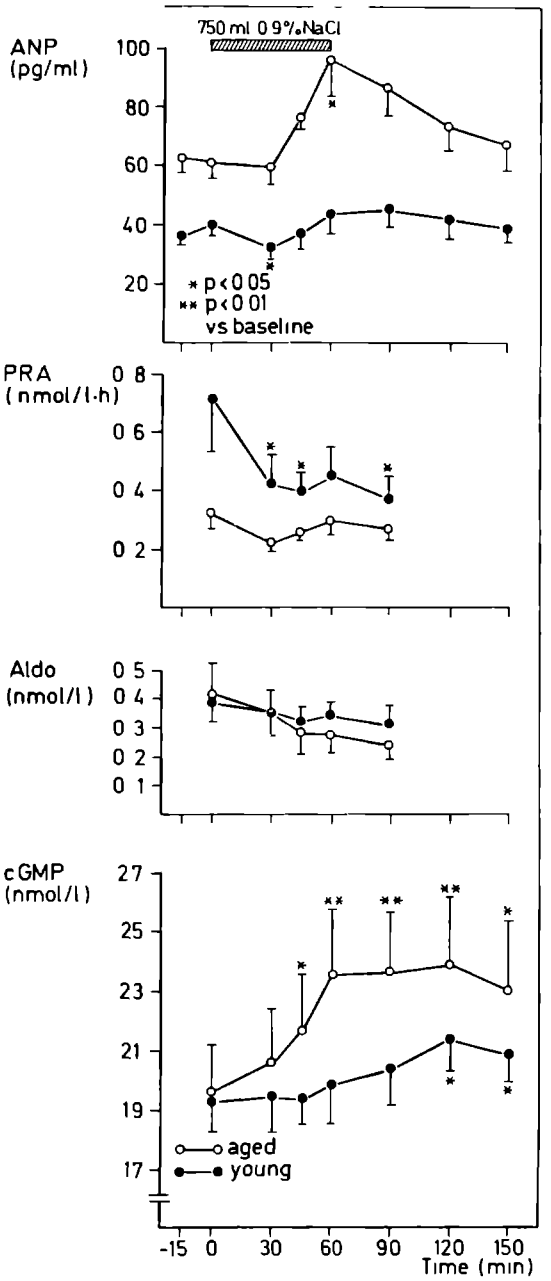

Figure IV-7. Time courses of ANP, PRA, aldosterone (Aldo) and cGMP during the volume loading study. Values are mean ± SEM. ANP significantly increased in the aged subjects while PRA decreased in the young subjects.

Table IV-3. Values obtained before (0 min), at the end of saline infusion (60 min), and 30 (90 min) and 90 (150) minutes thereafter. Values are expressed as mean ± SD. For abbreviations see text (TP = total protein).
* p<0.05 ** p<0.01 vs. values at 0 min.
\# p<0.05 young vs. aged subjects

		0 min	60 min	90 min	150 min
ANP	young	40.0 ± 10.3#	43.7 ± 18.2#	45.7 ± 18.0#	38.9 ±14.1#
	aged	61.4 ± 14.4	96.5 ± 34.3*	87.3 ± 27.9	67.8 ±23.5
PRA	young	0.71 ± 0.49#	0.46 ± 0.27*	0.37 ± 0.23*	-
	aged	0.33 ± 0.17	0.31 ± 0.16	0.27 ± 0.11	-
Aldo	young	0.39 ± 0.19	0.35 ± 0.12	0.31 ± 0.15	-
	aged	0.41 ± 0.33	0.28 ± 0.17	0.24 ± 0.14	-
cGMP	young	19.3 ± 7.3	19.9 ± 3.6	20.4 ± 3.5	20.9 ± 2.7*
	aged	19.6 ± 4.5	23.5 ± 6.8**	23.7 ± 5.7**	23.0 ± 6.9*
TP	young	65.8 ± 1.8	62.9 ± 1.4*	65.4 ± 2.7	-
	aged	65.6 ± 6.8	61.4 ± 6.5*	63.4 ± 5.2	-

occurred as shown by the values for total protein (table IV−3). Plasma volume expansion did not differ between the young (4.3 ± 2.4 %) and the elderly (6.4 ± 3.6 %) subjects. In neither group was the percentual change in ANP related to the change in plasma volume (young: r = 0.12, p = NS, aged: r = 0.48, p = NS). In the elderly subjects peak concentrations of ANP occurred at 66 ± 16 minutes after the start of the infusion whereas peak concentrations of cGMP were reached at 101 ± 32 minutes. The difference was statistically significant (p<0.05). For the young subjects, the corresponding values were 83 ± 21 minutes for ANP and 113 ± 35 minutes for cGMP (p = 0.12). The rise time to maximum ANP and cGMP concentrations between the two groups was not statistically significant.

Discussion

Ohashi *et al.* (43) infused hypertonic saline into young and aged men and observed an increase in both ANP and cGMP levels in the young as well as in the aged men. ANP rose from 11 to 36 pg/ml in the young and from 84 to 147 pg/ml in the aged men while cGMP rose similarly in both groups. Using isotonic saline solution at a dose insufficient to increase ANP levels in the young subjects, we observed a pronounced increase in the elderly subjects. The absence of a decrease in PRA in the elderly is in agreement with decreased responsiveness of the renin-aldosterone system with advancing age (44,45). The pronounced rise in ANP in the aged subjects can partly be explained by the decrease in clearance of ANP with advancing age (chapter 4.2). A given stimulus for secretion of ANP then results in an exaggerated and prolonged rise in ANP concentrations. Another explanation may be that the sensitivity of the ANP release system is increased in aged subjects. Indeed, Ruskoaho *et al.* (46) observed a greater increase in plasma ANP levels for a similar increase in right atrial pressure following volume expansion in aged rats as compared to young rats.

Aged subjects exhibit delayed natriuresis after saline infusion (47). One may speculate that this phenomenon causes a relative overfilling of the cardiovascular system in the aged subjects in comparison to the young subjects. As ANP release is governed by the filling of the intravascular volume one would then expect maximum ANP concentrations to be reached at a later time in the elderly. However, in our study the peak ANP levels occurred at 66 minutes after start of the infusion in the aged, and at 83 minutes in the young subjects. Furthermore volume expansion, as determined from the values for total protein, did not differ between the two groups. These results suggest that the delayed natriuresis in the elderly cannot explain the exaggerated ANP response in this group. Inversely, as peak concentrations of both ANP and cGMP occurred earlier in the elderly, this hormonal system does not seem to be responsible for this phenomenon.

One may wonder whether the exaggerated ANP response to a volume load in elderly subjects has a specific physiological significance. It may well be that this is a compensatory mechanism for decreased responsiveness of the target-organs. Indeed, although basal ANP levels were higher, basal cGMP levels in the elderly subjects were similar to those of the young subjects. Volume loading increased cGMP to a similar extent in both groups. As cGMP is considered to be the second messenger of ANP and cGMP concentrations in blood reflect the cellular response to ANP (40,41), we tentatively propose that with advancing age the responsiveness of the target-organs to ANP decreases. The ANP system functions at a higher level, both basal and after stimulation to compensate for this decreased

responsiveness. Consequently, elderly subjects possess a similar capacity of the atrial natriuretic peptide hormonal system to compensate for volume changes of the intravascular compartment.

Interestingly, volume loading did not significantly increase ANP levels in the young subjects but nevertheless induced a significant rise in cGMP levels. This may be an indication that even small changes in ANP concentrations exert biological effects. Furthermore the time lag between the rise in ANP and the rise in cGMP levels demonstrate that the maximal ANP levels as determined in blood samples drawn from a peripheral vein do not necessarily coincide with the maximal biological effect. This observation may also explain the time lag observed by Singer *et al.* (48) between the rise in ANP levels and the natriuresis and diuresis after volume loading.

In conclusion: the relationship between cardiac ANP secretion and advancing age was studied. Volume loading stimulated ANP only in the elderly subjects. Both the similar basal cGMP levels and the comparable increase during volume loading are indications of decreased responsiveness of the ANP target-organs with advancing age. This decreased responsiveness may be compensated by the higher ANP concentrations in elderly subjects, both in the basal state and after volume loading.

Chapter 4.5

Infusion of ANP

Introduction

As outlined in part I, the atrial natriuretic peptide (ANP) hormonal system is considered to function antagonistically to the renin-angiotensin-aldosterone system. When synthetic ANP is infused in humans a profound diuresis, natriuresis and blood pressure lowering effect is observed (49-54). Furthermore ANP suppresses renin release and aldosterone production, and exerts an inhibitory effect on the sympathetic nervous system (55-58). Many of these effects however, have been observed when synthetic ANP was infused in a high, possible pharmacological dose. It remains to be elucidated whether ANP can exert biologically important effects during low dose infusion. Therefore the haemodynamic and endocrine effects of (99-126)hANP infusion in both low dose and high dose were studied. The low dose was calculated to result in ANP values in the upper normal range while the high dose would result in ANP values comparable to those observed in disease states associated with chronic volume overload such as congestive heart failure (59,60).

In chapter 4.4, the effect of volume expansion on cardiac ANP secretion was compared in young and elderly subjects. Although ANP levels increased only in the aged group, cGMP values, reflecting biological effectiveness of ANP (38-41) rose to a similar extent thereby suggesting receptor down-regulation with diminished target organ responsiveness in the elderly. In order to assess directly whether ANP effector organs are down-regulated in elderly subjects, cGMP concentrations were measured during infusion of both low and high dose of synthetic (99-126)hANP. Furthermore the effects of ANP on the cardiovascular system and on various hormonal systems were compared. The study design was randomized, double-blind and placebo-controlled.

Methods and study design

Study design

The study protocol was approved by the local Hospital Ethical Committee. Two groups of healthy volunteers, 8 young (4 males, 4 females aged 18 to 25 years) and 8 elderly (4 males, 4 females aged 71 to 84 years) subjects recruited

from the general population, were studied. All subjects gave informed consent and were submitted to clinical examination before admission to the study. Physical examination revealed no cardiovascular, renal or endocrine disorders. None of the subjects smoked, used any drugs (oral contraceptives included) nor abused alcohol. The subjects were instructed to eat their usual diet, not to drink any coffee or tea and to refrain from hard labor during 24 hours preceding the study. (99-126)hANP was purchased from Bissendorf Peptide GmbH, Wedemark, FRG and dissolved in 48.0 ml Haemaccel (Behringwerke AG, Marburg, FRG) solution before use.

The studies were performed during three days separated by at least one week, in a climatized room (temperature: 24.6 ± 0.2°C, humidity: 54.7 ± 2.1 %, mean ± SD, n = 48). After having fasted for at least two hours, a catheter was inserted into an antecubital vein and placebo (Haemaccel) or (99-126)hANP was infused at random order in a double blind manner. The subjects remained in the supine position throughout the study period of 3 hours, including short periods for passing urine.

After one hour of acclimatization (supine position) either placebo or ANP (low dose: 0.25 μg/min, high dose: 2.0 μg/min) was infused during 60 minutes. In a preliminary study these infusion rates resulted in a two-fold rise of plasma ANP levels during the low, and in a twenty-fold rise during the high dose infusion. Dissolution of iodinated (99-126)hANP in Haemaccel resulted in 20 % loss of radioactivity to the infusion system, as compared to 50 % loss when ANP was dissolved in 0.9 % saline.

Haemodynamic measurements

Blood pressure was monitored with a semiautomatical arteriosonde. Electrocardiographic monitoring for assessment of heart rate was performed continuously. Forearm blood flow (FBF) was measured by venous occlusion plethysmography at 10 cm above heart level using mercury-in-rubber strain gauges. The skin circulation was excluded during FBF measurements by inflation of a wrist cuff to 50 mm Hg. The mean arterial pressure (MAP) was calculated as the sum of the diastolic pressure (DP) and 1/3 of the pulse pressure (systolic pressure (SP) - diastolic pressure). The forearm vascular resistance (FVR) was calculated as the quotient of MAP and FBF. Every 15 minutes two measurements were performed. The mean of these two measurements was used for data analysis. Haemoglobin and haematocrit values were measured just prior to infusion, at 45 and 60 minutes of infusion, and 60 minutes after termination of infusion.

Hormonal measurements

ANP was measured using the extraction method and the S-32 antibody, cGMP was measured as described in chapter 2.5. Plasma renin activity (PRA) was measured using the Phadebas Angiotensin I test (Pharmacia Diagnostics, Sweden), aldosterone (42), cortisol (61), adrenaline and noradrenaline (62) were measured as described previously. Blood was sampled from an antecubital vein (intravenous catheter inserted 60 minutes before infusion). On each study day approximately 120 ml blood was sampled. From one aged person blood could not be sampled on the placebo day. PRA, aldosterone, cortisol, adrenaline, noradrenaline and cGMP concentrations were measured in plasma obtained just prior to infusion (basal values), at 45 and 60 minutes of infusion and 30 minutes after infusion. ANP was measured in plasma obtained 30 minutes and just prior to infusion, at 15 (only high dose), 30, 45 and 60 minutes of infusion, and 15 (only high dose), 30 and 60 minutes after termination of infusion.

Statistical analysis

Results are expressed both as absolute values and as percentual changes from baseline values (haemodynamic data: the values measured just prior to infusion, hormonal data: concentrations assessed in blood obtained just prior to infusion, for ANP: mean of values in blood obtained 30 minutes and just prior to infusion). As plasma ANP values at 45 and 60 minutes of infusion did not differ during either infusion dose, the mean of haemodynamic and hormonal values measured at 45 and 60 minutes of infusion were considered to represent the values at steady state ANP infusion. As during placebo infusion significant changes did occur, the net percentual changes at steady state were calculated by subtracting the percentual change during placebo infusion from the corresponding percentual changes during low/high dose ANP infusion for each person.

Differences between basal and steady state values and between young and elderly subjects were studied using the Wilcoxon signed rank test and the Wilcoxon two-sample test. Significance was assessed at the $\alpha=0.05$ level. All values are expressed as mean ± SD, except where mentioned otherwise.

Results

In table IV−4 the group characteristics of the young and the elderly subjects are shown. Basal ANP levels were higher in the elderly while basal cGMP levels did not differ. During infusion of (99-126)hANP, immunoreactive ANP levels increased considerably in both groups reaching higher steady state values in the

Table IV-4. Group characteristics and humoral parameters measured under basal conditions. Values are mean ± SEM. ECC = endogenous creatinine clearance (calculated according to Cockcroft (5) and normalized for body surface area (6)). ANP and cGMP: basal values measured at the placebo infusion day. p = p-value young vs. aged: # $p<0.05$.

parameters		young (n=8)	p	aged (n=8)
Sex ratio	(M/F)	4/4		4/4
Age	(yr)	23.3 ± 1.0		75.7 ± 1.5
Height	(cm)	175.0 ± 3.1	NS	165.3 ± 4.5
Weight	(kg)	68.9 ± 5.5	NS	67.0 ± 4.5
Quetelet index	(kg/m^2)	22.4 ± 1.5	NS	24.5 ± 1.2
ECC	(ml/min/1.73 m^2)	109.6 ± 5.2	#	48.9 ± 4.7
ANP	(pg/ml)	34.5 ± 3.1	#	49.6 ± 4.4
cGMP	(nmol/l)	20.2 ± 1.8	NS	19.7 ± 2.0

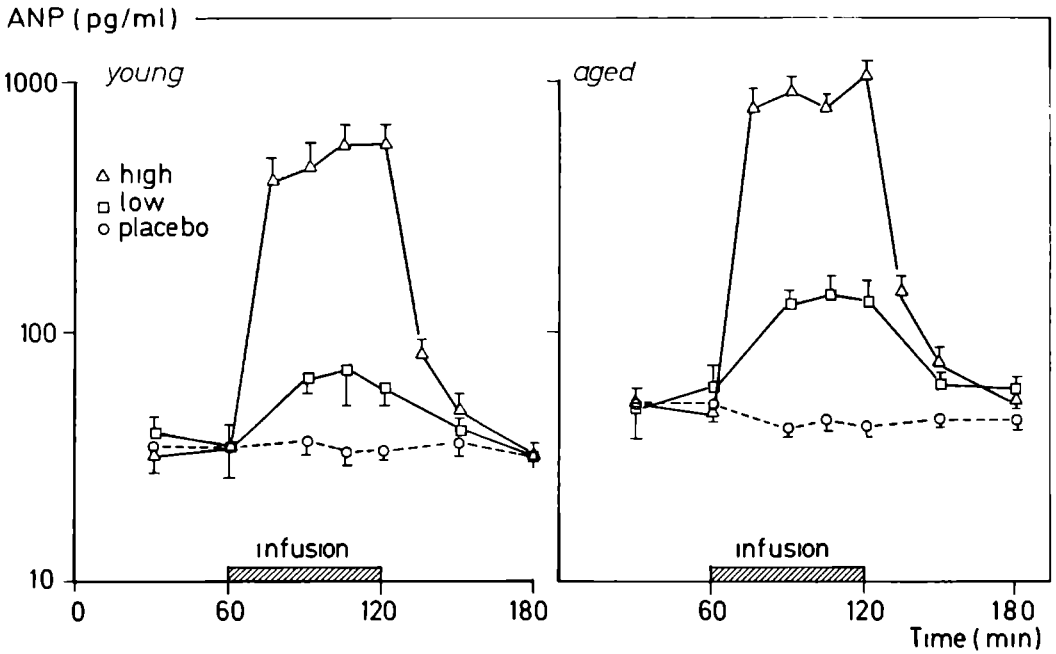

Figure IV-8. ANP concentrations during placebo, low dose (0.25 µg/min) and high dose (2.0 µg/min) infusion of (99-126)hANP in young (left panel) and aged (right panel) subjects. Values represent the mean ± SEM (n = 8).

elderly subjects. No change in ANP concentrations was observed during infusion of the vehicle alone (placebo infusion) (figure IV–8 and table IV-8).

Within-person analysis did not reveal a significant difference in basal values of either haemodynamic or hormonal parameter during the three study days. Between-group analysis indicated higher blood pressure and higher noradrenaline values in the elderly as compared to the young subjects.

Haemodynamic data

Absolute values are shown in table IV–5 and table IV–7. The percentual changes from baseline for the various haemodynamic parameters are shown in figure IV–9 and figure IV–10. As during placebo infusion significant changes did occur, the net percentual changes during low/high dose infusion are depicted in table IV–6. Following placebo and low dose infusion, mean arterial pressure (MAP) increased significantly in both young and aged subjects In the young, this was mainly due to an increase in diastolic pressure (DP), while both systolic pressure (SP) and DP increased in the elderly Heart rate did not change significantly. Net percentual changes during the low dose infusion were not significant. In contrast, during high dose infusion a significant blood pressure lowering effect was observed in both groups, while heart rate increased in the elderly. The decrease in MAP was more pronounced in the elderly, mainly due to an exaggerated decrease in SP. Correspondingly, heart rate increased in both groups but the increase was significant only in the aged group

Forearm blood flow (FBF) decreased during placebo and low dose infusion, while it increased at high dose The net percentual change was significant only in the elderly subjects Forearm vascular resistance (FVR) being the quotient of MAP and FBF, exhibited an inverse pattern and increased at placebo and low dose infusion and decreased at high dose infusion In figure IV–11 the net percentual time curves (with placebo time curves subtracted) for forearm vascular resistance and mean arterial pressure are given.

Haemodynamic effects had a relatively rapid onset of action, as effects were observed shortly (within 15 minutes) after start of high dose ANP infusion, consisting of a decrease in SP, DP, MAP and FVR with an increase in FBF and HR. As within-group variation varied considerably, only the net percentual changes in SP and MAP were significantly greater in the aged group as compared to the young group

During placebo infusion and low dose infusion, haematocrit and haemoglobin values did not change in the elderly while they increased at low dose in the young (figure IV–12). At high dose infusion, haematocrit and haemoglobin increased in both groups. Although the increase was more pronounced in the el-

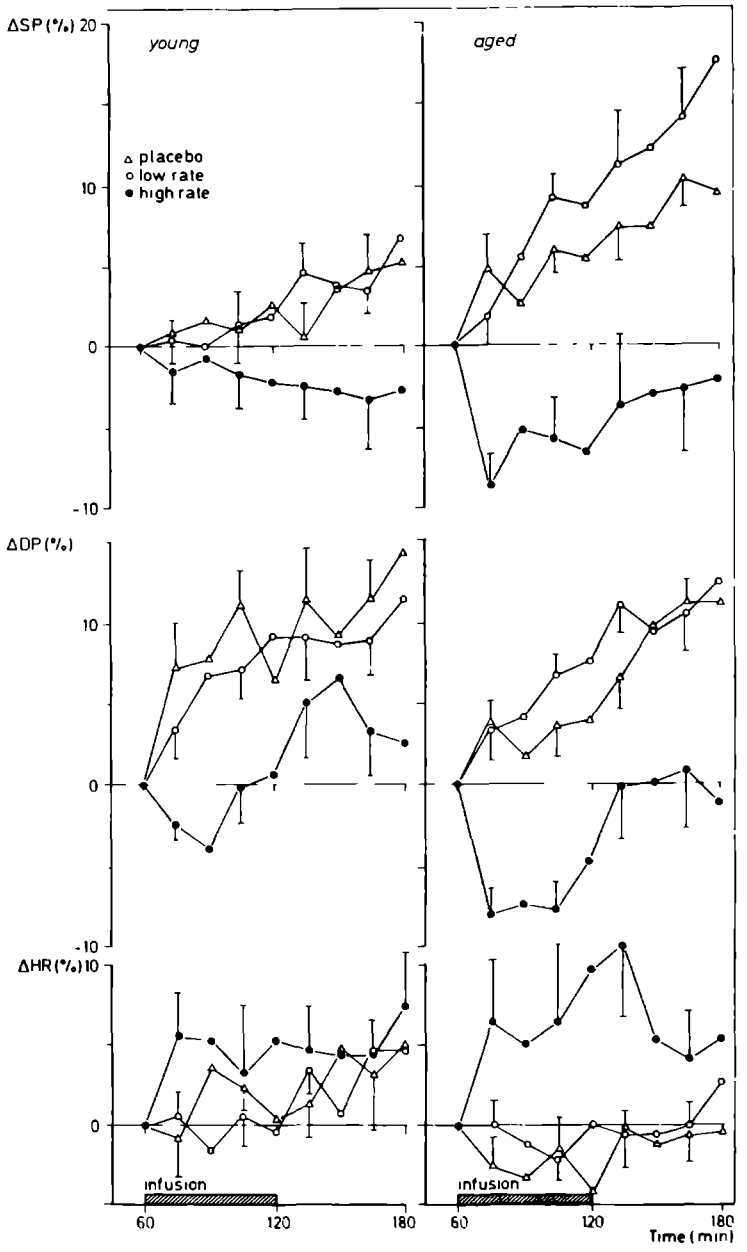

Figure IV-9. Percentual changes in systolic pressure (SP), diastolic pressure (DP) and heart rate (HR) during infusion of placebo (△—△), low (○—○) or high (●—●) rate ANP in young (left panel) and aged (right panel) subjects. Values are mean ± SEM (n = 8).

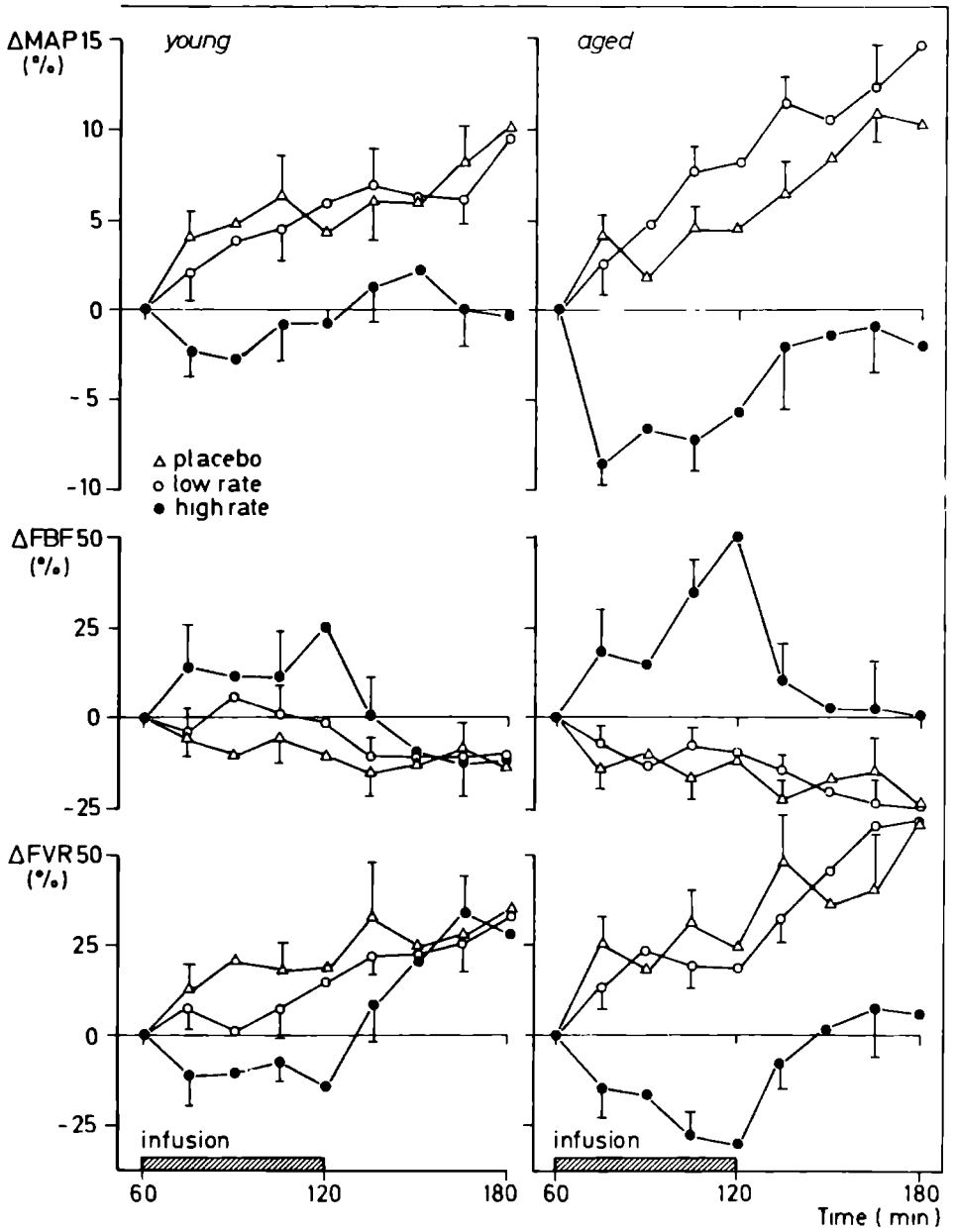

Figure IV-10. Percentual changes in mean arterial pressure (MAP), forearm blood flow (FBF) and forearm vascular resistance (FVR) during infusion of placebo (△—△), low (○—○) or high (●—●) rate ANP in young (left panel) and aged (right panel) subjects. Values are mean ± SEM (n = 8).

Table IV-5. Effects of high dose (H), low dose (L) or placebo (P) infusion on systolic arterial pressure (SP, mm Hg), diastolic arterial pressure (DP, mm Hg), mean arterial pressure (MAP, mm Hg), heart rate (HR, beats/min), forearm blood flow (FBF, ml/100 ml/min) and forearm vascular resistance (FVR, U) in young (n=8) and aged (n=8) subjects before, during (mean of values at 45 and 60 minutes of infusion) and after infusion (mean of values at 45 and 60 minutes after infusion). Values are mean ± SD.

* $p<0.05$ vs. baseline

		young			aged		
		before	during	after	before	during	after
SP	P	111 ±15	112 ±15	115 ±13	137 ±14	145 ±17*	151 ±16*
	L	110 ±11	111 ±12	115 ±14*	133 ±17	145 ±23*	155 ±23*
	H	113 ±11	110 ±10	109 ±12	138 ±15	129 ±15*	134 ±17
DP	P	66 ±7	71 ±7*	74 ±6*	77 ±10	80 ±11	86 ±12*
	L	68 ±7	74 ±6*	75 ±6*	77 ±9	83 ±10*	86 ±10*
	H	71 ±9	71 ±6	72 ±6	80 ±11	75 ±11*	80 ±12
MAP	P	81 ±9	85 ±9	88 ±8*	97 ±10	102 ±12*	108 ±13*
	L	82 ±10	86 ±8*	89 ±8*	96 ±11	103 ±13*	109 ±14*
	H	85 ±8	84 ±6	85 ±6	99 ±10	93 ±11*	98 ±12
HR	P	70 ±6	71 ±7	73 ±11	62 ±10	60 ±10	62 ±10
	L	68 ±8	68 ±9	72 ±11	61 ±11	60 ±10	62 ±10
	H	74 ±7	77 ±13	78 ±10	62 ±11	67 ±12*	65 ±11
FBF	P	7.8 ±6.6	6.8 ±5.7	6.4 ±5.2	6.3 ±3.4	5.3 ±2.9	4.8 ±3.1
	L	6.4 ±2.6	6.2 ±2.8	5.3 ±1.8*	7.1 ±3.2	6.6 ±3.2	5.2 ±2.5*
	H	7.9 ±6.2	8.1 ±4.8	5.8 ±3.6	6.7 ±3.5	8.9 ±3.6*	6.9 ±4.2
FVR	P	17 ±10	19 ±11	20 ±10*	21 ±14	27 ±19*	30 ±15*
	L	15 ±8	17 ±9	19 ±9	16 ±6	19 ±8*	25 ±10*
	H	18 ±11	14 ±7	21 ±13	18 ±8	12 ±4*	19 ±11

Table IV-6. Baseline values and net percentual changes (mean of values at 45 and at 60 minutes of infusion) of systolic arterial pressure (SP, mm Hg), diastolic arterial pressure (DP, mm Hg), mean arterial pressure (MAP, mm Hg), heart rate (HR, beats/min), forearm blood flow (FBF, ml/100 ml/min), forearm vascular resistance (FVR, U), haemoglobin (Hg, mmol/l) and haematocrit (Ht, l/l) values during low dose and high dose infusion of ANP in young (n=8) and aged (n=8) subjects. Percentual changes during placebo infusion are subtracted from percentual changes during low/high dose infusion. Values are mean ± SD.
p = p-value # $p<0.05$ young vs. aged
* $p<0.05$ vs. baseline

	Low dose - placebo			High dose - placebo		
	young	p	aged	young	p	aged
SP basal	110 ±11		133 ±17	113 ±11		138 ±15
% change	-0.0 ±9.6	NS	3.3 ±4.8	-3.7 ±3.6*	#	-11.9 ±6.6*
DP basal	68 ±7		77 ±9	71 ±9		80 ±11
% change	-0.6 ±8.0	NS	3.5 ±6.2	-8.4 ±7.1*	NS	-10.1 ±4.8*
MAP basal	82 ±10		96 ±11	85 ±8		99 ±10
% change	-0.2 ±8.1	NS	3.4 ±4.7	-6.2 ±4.5*	#	-11.0 ±4.1*
HR basal	68 ±8		61 ±11	74 ±7		62 ±11
% change	-1.1 ±5.6	NS	1.8 ±12.6	3.2 ±8.7	NS	11.0 ±11.3*
FBF basal	6.4 ±2.6		7.1 ±3.2	7.9 ±6.2		6.7 ±3.5
% change	8.7 ±38.9	NS	6.1 ±24.3	27.4 ±36.0	NS	56.8 ±41.9*
FVR basal	15 ±8		16 ±6	18 ±11		18 ±8
% change	-7.7 ±37.0	NS	-8.7 ±32.7	-29.6 ±27.8*	NS	-57.7 ±32.6*
Hb basal	7.95 ±0.73		7.99 ±0.90	8.45 ±0.79		7.89 ±0.67
% change	3.3 ±3.8*	NS	3.0 ±6.45	4.9 ±2.7*	NS	8.8 ±12.2
Ht basal	0.38 ±0.03		0.39 ±0.03	0.39 ±0.03		0.38 ±0.03
% change	4.0 ±3.9*	NS	1.0 ±4.6	6.6 ±2.3*	NS	9.6 ±10.7*

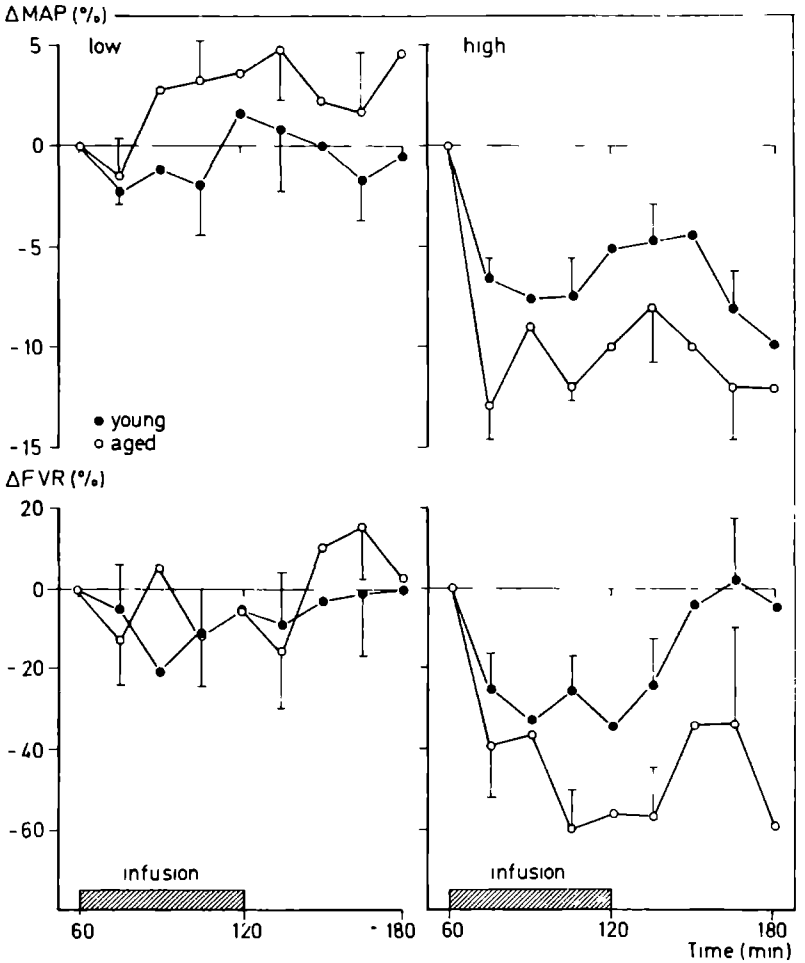

Figure IV-11. Net percentual time curves for forearm vascular resistance (FVR) and mean arterial pressure (MAP) at low dose (left panel) and high dose (right panel) infusion of ANP in young (●—●) and aged (○—○) subjects. Values are mean ± SEM (n = 8).

derly, due to the large within-group variation in the elderly the net percentual changes in haemoglobin were significant only in the young subjects.

Table IV-7. Effects of high dose (H), low dose (L) or placebo (P) infusion on haemoglobin (Hb, mmol/l) and haematocrit (Ht, l/l) values in young (n=8) and aged (n=8) subjects before, during (mean of values at 45 and 60 minutes of infusion) and after infusion (60 minutes after infusion). Values are mean ± SD.
* $p<0.05$ vs. baseline

		young			aged		
		before	during	after	before	during	after
Hb	P	8.05 ±0.63	8.13 ±0.59	8.15 ±0.54	8.17 ±0.31	8.11 ±0.71	7.90 ±0.65
	L	7.95 ±0.73	8.29 ±0.7*	8.38 ±0.82*	7.99 ±0.90	8.08 ±0.88	8.26 ±0.63
	H	8.45 ±0.79	8.93 ±0.60*	8.78 ±0.90	7.89 ±0.67	8.53 ±0.47*	8.41 ±0.56*
Ht	P	0.39 ±0.02	0.38 ±0.02	0.38 ±0.02	0.38 ±0.02	0.37 ±0.03	0.38 ±0.03
	L	0.38 ±0.03	0.39 ±0.03*	0.39 ±0.03*	0.39 ±0.03	0.39 ±0.03	0.39 ±0.03
	H	0.39 ±0.03	0.42 ±0.02*	0.41 ±0.03*	0.38 ±0.03	0.41 ±0.02*	0.40 ±0.02*

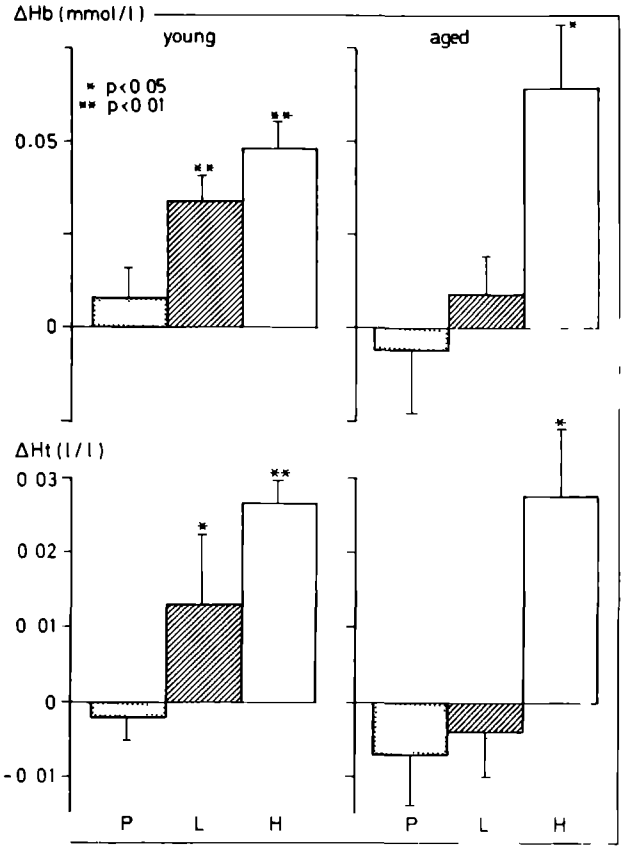

Figure IV-12. Net changes in haemoglobin (Hb) and haematocrit (Ht) values during infusion of placebo (P), low (L) or high (H) dose ANP infusion in young (left panel) and aged (right panel) subjects. Values are mean ± SEM (n = 8).

Hormonal data

Figures IV−13 and IV−14 represent percentual changes in hormonal concentrations, while absolute values are shown in table IV−8. The net percentual changes during low/high dose ANP infusion are shown in table IV-9.

PRA decreased during placebo and low dose infusion in both groups while no change occurred during high dose ANP infusion. When subtracting the percentual change during placebo infusion from the percentual change during high dose infusion, a relative increase in PRA was observed. Due to the large inter-individual variation, the difference was not significant. Plasma aldosterone and cortisol did not change in the young, but aldosterone decreased during high dose infusion in the el-

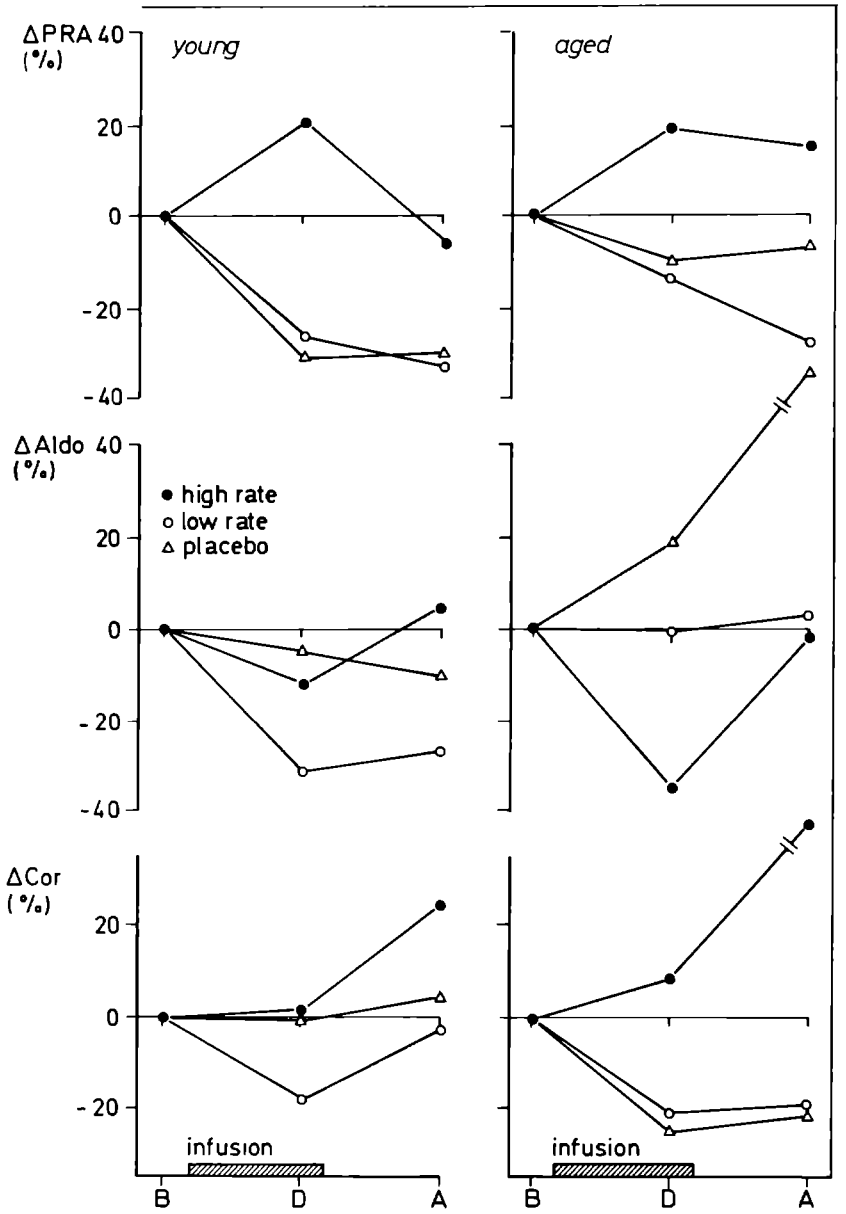

Figure IV-13. Percentual changes in plasma renin activity (PRA), aldosterone (Aldo) and cortisol (Cor) before (B), during (D) and after (A) infusion of placebo (△ — △), low (○ — ○) or high (● — ●) rate ANP in young (n = 8, left panel) and aged (n = 8, right panel) subjects. For absolute values, see table IV-8.

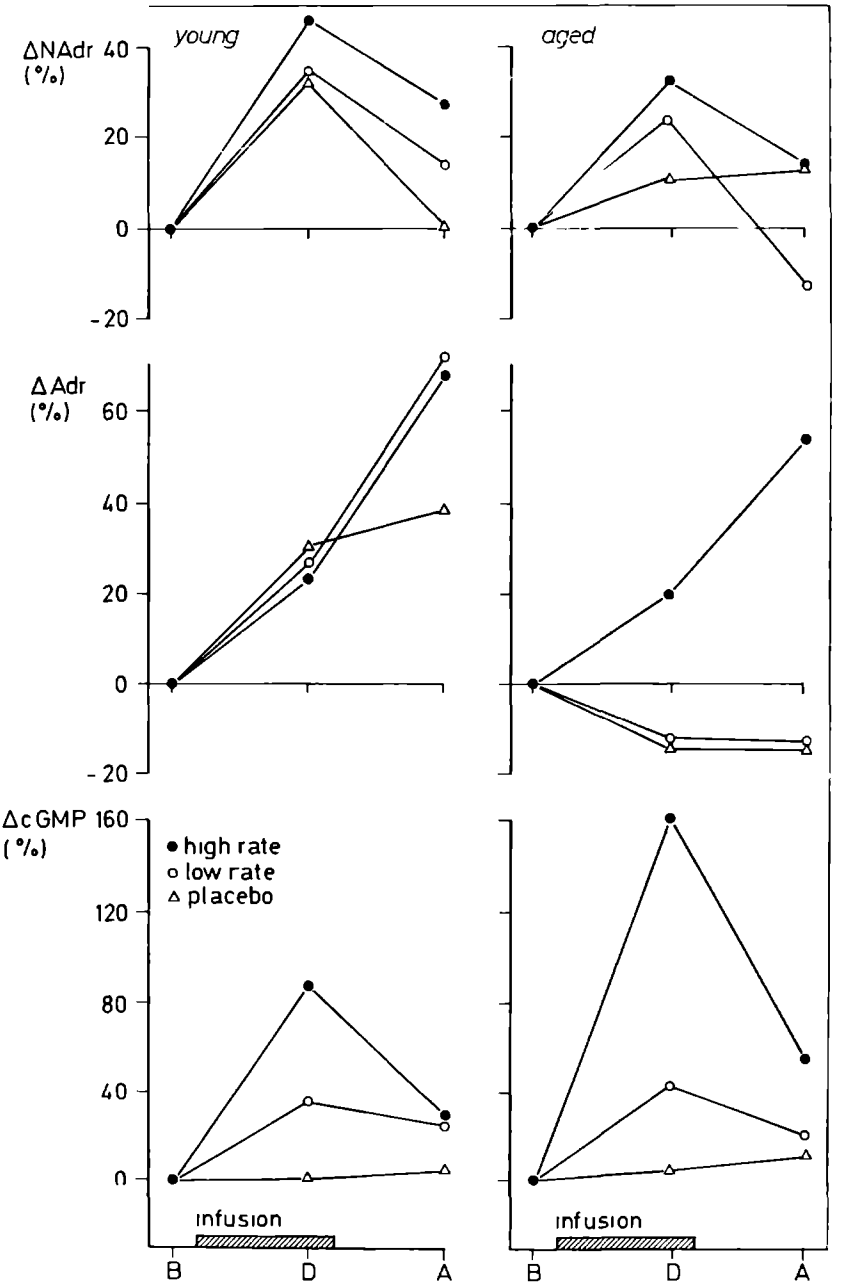

Figure IV-14. Percentual changes in noradrenaline (NAdr), adrenaline (Adr) and cyclic guanosine monophosphate (cGMP) before (B), during (D) and after (A) infusion of placebo (△—△), low (○—○) or high (●—●) rate ANP in young (n = 8, left panel) and aged (n = 8, right panel) subjects. For absolute values, see table IV–8.

Table IV-8. Effects of high dose (H), low dose (L) or placebo (P) infusion on plasma levels of PRA (nmol/l/h), aldosterone (Aldo, nmol/l), cortisol (Cor, µmol/l), adrenaline (Adr, nmol/l), noradrenaline (NAdr, nmol/l), cGMP (nmol/l) and ANP (pg/ml) in young (n=8) and aged (n=8) subjects before, during (mean of values at 45 and 60 minutes of infusion) and after infusion (30 minutes after infusion). Values are mean ± SD. * $p<0.05$ vs. baseline

		young			aged		
		before	during	after	before	during	after
	P	0.87 ±0.48	0.59 ±0.32*	0.52 ±0.27*	0.51 ±0.41	0.41 ±0.25*	0.41 ±0.20
PRA	L	0.57 ±0.35	0.41 ±0.24*	0.37 ±0.24*	0.54 ±0.37	0.41 ±0.21	0.34 ±0.17*
	H	0.98 ±0.54	1.02 ±0.55	0.78 ±0.36	0.55 ±0.42	0.56 ±0.29	0.51 ±0.25
	P	0.39 ±0.28	0.30 ±0.18	0.26 ±0.12	0.25 ±0.15	0.28 ±0.25	0.37 ±0.36
Aldo	L	0.53 ±0.35	0.30 ±0.09	0.31 ±0.13	0.31 ±0.16	0.25 ±0.09	0.24 ±0.06
	H	0.45 ±0.25	0.34 ±0.18	0.33 ±0.16	0.37 ±0.18	0.23 ±0.15*	0.37 ±0.36
	P	0.21 ±0.10	0.20 ±0.09	0.20 ±0.08	0.21 ±0.07	0.16 ±0.06*	0.16 ±0.06
Cor	L	0.27 ±0.11	0.20 ±0.06	0.20 ±0.10	0.21 ±0.05	0.16 ±0.03*	0.17 ±0.08
	H	0.24 ±0.08	0.22 ±0.08	0.26 ±0.11	0.20 ±0.06	0.20 ±0.06	0.35 ±0.24
	P	0.09 ±0.04	0.11 ±0.05	0.12 ±0.07	0.14 ±0.07	0.11 ±0.05	0.11 ±0.04
Adr	L	0.09 ±0.04	0.10 ±0.04	0.15 ±0.07	0.13 ±0.07	0.11 ±0.07	0.12 ±0.07
	H	0.11 ±0.06	0.11 ±0.03	0.16 ±0.04	0.12 ±0.05	0.13 ±0.10	0.18 ±0.13
	P	0.69 ±0.17	0.89 ±0.21*	0.68 ±0.18	1.50 ±0.37	1.64 ±0.67	1.69 ±1.19
NAdr	L	0.64 ±0.13	0.79 ±0.26	0.71 ±0.19	1.18 ±0.51	1.43 ±0.57*	1.10 ±0.82
	H	0.90 ±0.36	1.17 ±0.54	1.00 ±0.47	1.53 ±0.62	1.86 ±0.65	1.63 ±0.46
	P	20.2 ±5.1	19.9 ±3.3	21.1 ±5.7	19.7 ±5.7	20.0 ±3.3	21.5 ±4.5
cGMP	L	20.2 ±5.9	26.7 ±4.8*	23.8 ±4.1	18.6 ±2.8	26.6 ±3.3*	22.2 ±3.3*
	H	21.4 ±2.5	39.8 ±6.8*	27.5 ±3.8*	20.8 ±4.1	54.5 ±14.3*	35.6 ±10.6*
	P	34.5 ±8.9	33.1 ±11.7	31.1 ±12.7	49.6 ±12.5	43.8 ±9.4	44.9 ±10.6
ANP	L	37.2 ±28.3	75.8 ±38.4*	33.7 ±8.2	54.9 ±31.3	132.7 ±65.4*	55.3 ±65.4
	H	35.1 ±17.7	570 ±237*	32.7 ±4.1	49.6 ±14.0	1001 ±429*	53.8 ±13.3

Table IV-9. Baseline values and net percentual changes (mean of values at 45 and at 60 minutes of infusion) in plasma renin activity (PRA, nmol/l/h), aldosterone (Aldo, nmol/l), cortisol (Cor, µmol/l), adrenaline (Adr, nmol/l), noradrenaline (NAdr, nmol/l), cGMP (nmol/l) and ANP (pg/ml) values during low dose and high dose infusion of ANP in young (n=8) and aged (n=7) subjects. Percentual changes during placebo infusion are subtracted from percentual changes during low/high dose infusion. Values are mean ± SD.
p = p-value # $p<0.05$ young vs. aged
* $p<0.05$ vs. baseline

	Low dose - placebo			High dose - placebo		
	young	p	aged	young	p	aged
PRA basal	0.57 ±0.35		0.54 ±0.37	0.98 ±0.54		0.94 ±1.46
% change	3.8 ±17.3	NS	2.6 ±23.0	50.8 ±75.8	NS	26.6 ±26.9
Aldo basal	0.53 ±0.35		0.31 ±0.16	0.45 ±0.25		0.37 ±0.18
% change	-27.9 ±51.2	NS	-11.7 ±71.1	-7.6 ±31.2	NS	-50.9 ±42.3*
Cor basal	0.27 ±0.11		0.21 ±0.05	0.24 ±0.08		0.20 ±0.06
% change	-18.2 ±45.3	NS	4.0 ±23.4	1.9 ±83.2	NS	37.1 ±55.1
Adr basal	0.09 ±0.04		0.13 ±0.07	0.11 ±0.06		0.12 ±0.05
% change	-3.9 ±72.5	NS	0.1 ±27.6	-7.1 ±38.8	NS	27.0 ±109.7
NAdr basal	0.64 ±0.13		1.18 ±0.51	0.90 ±0.36		1.53 ±0.62
% change	2.6 ±67.6	NS	10.9 ±41.0	14.4 ±93.1	NS	0.8 ±54.1
cGMP basal	20.2 ±5.9		18.6 ±2.8	21.4 ±2.5		20.8 ±4.1
% change	37.3 ±16.4*	NS	38.1 ±13.6*	86.4 ±32.7*	#	144.6 ±43.9*
ANP basal	37.2 ±28.3		54.9 ±31.3	35.1 ±17.7		49.6 ±14.0
% change	136.6 ±31.0*	NS	163.3 ±37.5*	1723 ±1033*	NS	2044 ±872*

derly while cortisol remained constant. During low and placebo infusion however, cortisol values decreased significantly in the elderly subjects. Net percentual changes revealed only a decrease in aldosterone in the elderly during high dose infusion.

Adrenaline did not exhibit consistent changes neither in the young nor in the elderly at either infusion dose. Noradrenaline increased at steady state low dose infusion in the elderly and at steady state placebo infusion in the young subjects. No

significant net percentual changes neither at low dose nor at high dose ANP infusion were observed for both adrenaline and noradrenaline.

Basal plasma concentrations of the second messenger of the ANP hormonal system, cyclic guanosine monophosphate (cGMP) were similar and increased during low dose ANP infusion to a comparable extent in young and aged subjects. When placebo was infused, no change in cGMP values was observed in agreement with the lack of change in ANP concentrations. During high dose ANP infusion, the cGMP increase was more pronounced in the elderly subjects.

Side effects

With regard to the diuretic effects of ANP, no urgency was observed during or in the first 30 minutes after discontinuation of placebo infusion. Following low dose infusion, urgency was perceived in 5 subjects (1 elderly, 4 young), following high dose infusion urgency was perceived in 10 subjects (4 elderly, 6 young) When urgency was experienced the subjects micturated instantly in the supine position. During low/high dose infusion of ANP no hypotension, light-headedness or bradycardia were observed.

Discussion

The higher basal ANP concentrations in elderly subjects observed in this study confirm the observations reported in chapter 4.1 that ANP concentrations increase with advancing age During infusion of both low and high dose of (99-126)hANP, the plasma concentrations reached at steady state were markedly higher in the elderly subjects as compared to the young subjects. This cannot be attributed to differences in height, weight or quetelet-index as these parameters were similar in both groups. The only difference was the lower endogenous creatinine clearance in the aged group As the total amount of (99-126)hANP infused was similar in both groups, the higher ANP concentrations reached in the elderly subjects can only be explained by a diminished clearance of ANP as compared to the young subjects (chapter 4.2).

Haemodynamic data

Low dose ANP infusion resulted in immunoreactive ANP concentrations within the physiological range. During this infusion dose no significant effects of ANP on cardiovascular parameters (blood pressure, heart rate, forearm blood flow) were observed. Other investigators, performing ANP infusion studies in humans, reported a potent blood-pressure lowering effect (49-54). The ANP concentrations reached

during these infusion studies however, far exceeded the normal range. In our study, low dose ANP infusion resulted in only slightly elevated ANP levels. Richards *et al.* (63), using low dose and intra-arterial blood pressure monitoring, observed a decrease in mean arterial pressure. This observation was confirmed by Shenker *et al.* (64). In contrast, Solomon *et al.* (65) were not able to demonstrate significant changes in heart rate or blood pressure. These investigators however, infused low dose ANP for a period up to several hours. It may well be that when ANP is infused at a dose sufficient to raise plasma levels only slightly, a long infusion period is necessary to exert biological effects while at high dose ANP infusion, effects are more rapid in onset.

At high dose infusion, ANP decreased mean arterial pressure (MAP) in both groups, with a more pronounced decrease in the elderly. Although heart rate, as expected from the fall in MAP, increased in both groups, the increase was significant only in the elderly subjects. With blood pressure falling by an average of 6 to 11 % at high dose infusion, one would expect activation of the sympathetic nervous system resulting in reflex tachycardia. However, one should take into account that these are net percentual changes. The net percentual decrease in blood pressure at high dose resulted mainly from an increase during placebo infusion. In absolute values only a minor decrease in MAP was observed, which was significant only in the elderly.

At high dose infusion, as expected from the vasodilator potency of ANP, forearm blood flow (FBF) increased and forearm vascular resistance (FVR) decreased. This is consistent with reports from other investigators (66-68). At low dose infusion, no change neither in FBF nor in FVR was observed. This contrasts with the findings of Bolli *et al.* (68) and Hughes *et al.* (66). These investigators however infused low dose ANP intra-arterially. Regional ANP levels and effects achieved with intra-arterial infusion cannot be compared with those achieved with intravenous infusion.

In view of the vasodilating potency of ANP (66,69), one might expect an increase in intravascular volume with a concomitant decrease in haemoglobin and haematocrit. Instead, consistent with other reports (63,65,70) an increase in haemoglobin and haematocrit was observed during infusion of ANP, suggesting a decrease in intravascular volume. It has to be noted however, that in absolute values, FVR decreased only during high dose in the elderly. Furthermore, FVR may not accurately reflect total peripheral resistance (TPR). Because in our study cardiac output was not measured, TPR could not be calculated. Results from studies regarding TPR after infusion of ANP in healthy humans have not been consistent. TPR was shown to remain constant (71), to decrease (70,72) or even to increase (73,74). These inconsistent results can partly be attributed to the molecular form of ANP infused, the dose of ANP given, the mode of administration (e.g. bolus or constant intravenous infusion, or both), the duration of infusion and whether or not the study was

placebo-controlled.

In rats, blood volume decreased following infusion of ANP (75,76). This decrease in blood volume cannot be attributed solely to increased diuresis since this phenomenon was also observed in anephric rats (77,78). ANP induces fluid shift from the intravascular fluid compartment to the interstitial space, presumably by increasing capillary hydraulic conductivity, increasing vascular permeation of proteins and elevating postcapillary resistance (75,76,79). Trippodo *et al.* (75) found that ANP decreased circulatory capacitance in rats. This was attributed to a decrease in blood volume and to active venoconstriction, resulting in diminished venous return to the heart. Indeed, infusion of ANP in humans decreases central venous pressure (73,80). Reducing preload decreases cardiac output, which in turn decreases arterial pressure. Therefore the blood pressure lowering effect of ANP can be attributed to both a decrease in TPR and a reduction in blood volume, which by decreasing venous return, diminishes cardiac output.

Comparison of the cardiovascular data revealed a larger net percentual decrease in MAP during the high dose infusion in the elderly subjects. This difference was largely due to the more pronounced fall in SP. However, the ANP concentrations reached during this dose were far higher in the elderly. In view of the more pronounced increase in cGMP values at this dose, more exaggerated effects of ANP in the aged group may not come as a surprise.

Since significant volume contraction as shown by the haematocrit and haemoglobin values occurred at low dose in the young but not in the elderly subjects, one can speculate on receptor down-regulation with diminished target-organ responsiveness in the elderly. Considering the greater sensitivity to ANP in promoting volume contraction in the young while the cardiovascular parameters were not measurably influenced at low dose ANP, one can hypothesize that with advancing age the reducing effect on intravascular volume is preferentially down-regulated. As elderly subjects are more prone to dehydration (32,81), it may be that this is a functional mechanism by which the body protects the volume of the intravascular compartment. In this respect it would be interesting to compare the renal effects of ANP in young and elderly subjects.

Summarizing, ANP at low dose did not exert significant cardiovascular effects but instead induced volume contraction. Since Solomon *et al.* (65) did not observe any cardiovascular effects but instead a diuresis and natriuresis, it may well be that at low dose ANP exerts its effect on the cardiovascular system primarily by reducing the volume of the intravascular compartment (diuresis, natriuresis, extravasation of fluid). With higher doses ANP also exerts direct effects on the cardiovascular system (vasorelaxation, blood pressure lowering and hormonal effects).

Although both basal and steady state ANP concentrations at low dose ANP infusion were markedly higher in the elderly, basal and steady state concentrations of its second messenger cGMP were similar in both groups. Only during high dose infusion, when mean ANP levels were higher than 1000 pg/ml in the elderly as compared to 570 pg/ml in the young, did the cGMP response differ Because assessment of cGMP plasma levels reflect the response of the target-organs for ANP (40,41), this is an indication that with advancing age a down-regulation of ANP target-organ responsiveness occurs This decreased responsiveness however is offset by the higher ANP concentrations reached during infusion of ANP.

In vitro, ANP has been reported to inhibit renin release from juxtaglomerular cells, aldosterone and cortisol production from cultured adrenal cells and norepinephrine release from adrenal pheochromocytoma cells (55,56,82,83). Indeed, infusion of ANP resulted in some (84,85) but not all studies (49,73), in a decrease in PRA, aldosterone, cortisol, and catecholamines Some investigators even observed an increase of PRA and catecholamines (86,87). This may be due to the potent blood pressure lowering effect of ANP resulting in reflex stimulation of the sympathetic nervous system. Although it seems that ANP exerts some inhibitory effect on the sympathetic nervous system (57,88,89), with high dose of ANP the fall in blood pressure may trigger a strong sympathetic reflex overriding the inhibitory effect of ANP on the sympathetic system (73,90)

The above mentioned effects were observed when rather high doses of ANP (varying from 2 μg/min to 12 μg/min, with or without prior bolus injection of ANP) were given The plasma concentrations of immunoreactive ANP reached during these high dose infusions far exceed the normal range, thereby raising doubt whether these ANP induced effects are physiological or rather pharmacological by nature Therefore, (99-126)hANP was infused in low dose (0 25 μg/min) resulting in concentrations within the physiological range. Steady state ANP concentrations were reached after 45 minutes of infusion. Because during placebo infusion significant changes did occur, the percentual changes from baseline during placebo infusion were subtracted from the percentual changes during low dose infusion. However, no significant changes in PRA, aldosterone, cortisol or catecholamines was observed. These results suggest that ANP infused at low though biologically active dose as shown by the increase in cGMP values, does not exert significant effects on these hormonal systems neither in young nor in elderly persons. Recently several investigators reported that even at low dose ANP infusions (0.16 to 0 4 μg/min) a natriuresis, diuresis and blood pressure lowering effect could be demonstrated (63-65). Also, PRA and aldosterone levels decreased These effects however were

reached only during infusion of low dose ANP for several hours while in our study infusion was continued for only 60 minutes.

During infusion of high dose ANP plasma levels were elevated far above the normal range and were similar to ANP concentrations found in congestive heart failure (59,60). This resulted in a relative increase in PRA in both young and elderly subjects. This increase however was not significant. In absolute values, no change in PRA occurred during high dose infusion of ANP.

In contrast to the rise in PRA, plasma aldosterone fell sharply in the elderly but not in the young subjects. This phenomenon can be explained by the observed inhibition of both basal and stimulated aldosterone production by ANP (58,82,91). The more pronounced decrease in net percentual change in aldosterone levels in the elderly can be attributed to greater sensitivity of the adrenal in these subjects or, more likely, to the more pronounced biological effect of ANP as reflected in the higher cGMP levels reached. As for plasma cortisol, no significant change occurred in the young at either infusion mode. In the elderly cortisol did not change at high dose infusion whereas at placebo and low rate infusion cortisol decreased in the elderly. The net percentual change in cortisol at high dose ANP however was, due to the large inter-individual variation, not significant. No changes in plasma concentrations of adrenaline were observed in either group indicating that ANP at this dose does not modify the secretion of adrenaline from the adrenal medulla.

During high dose infusion of ANP, only a minor decrease in MAP was observed. This resulted in slight stimulation of the sympathetic nervous system, as shown by the small increase in heart rate. These changes were significant only in the elderly. In these subjects, noradrenaline values tended to increase although this increase was not significant. However, it can be questioned whether peripheral venous noradrenaline concentrations accurately reflect the activity of the sympathetic nervous system, especially when only minor changes in MAP and HR are observed. Furthermore it has been shown that ANP inhibits noradrenaline release (92,93). Ebert *et al.* (88) observed an attenuation of carotid baroreflex-mediated cardioacceleration by ANP in humans. These observations suggest that ANP may modulate the sympathetic nervous system. In view of the above mentioned facts, the lack of consistent changes in plasma levels of noradrenaline may not be surprising.

In conclusion: the cardiovascular and hormonal effects of ANP were assessed during infusion of (99-126)hANP at low and at high dose. Furthermore a comparison was made between young and elderly subjects. In general, low dose infusion of ANP did not exert major cardiovascular or hormonal effects in either group. High dose infusion induced a fall in blood pressure and forearm blood flow, and increased heart rate. With regard to the hormonal parameters, only aldosterone decreased

significantly. In general, the effects of high dose infusion were more pronounced in the elderly group, which is reflected in the higher cGMP values (a measure of the biological effect of ANP) reached. The ANP concentrations reached at high dose however, were far higher in the elderly. Considering the similar basal cGMP values, the similar increase in cGMP at low dose infusion and the haemoconcentration at low dose occurring only in the young, we propose that down-regulation of ANP target-organ responsiveness occurs with advancing age. This diminished responsiveness however is compensated by an exaggerated increase in ANP concentrations during infusion of ANP.

Chapter 4 6
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Part V

STUDIES OF ANP IN PATHOPHYSIOLOGICAL STATES

In the preceding parts of this thesis a method for assessment of ANP concentrations in human blood was developed and evaluated. In this part the method developed was applied to two disease states in which the atrial natriuretic peptide hormonal system may be involved. In general, the release of ANP is determined by the volume of the intravascular system. However, as ANP secretion is directly related to the degree of stretch of the atrial myocytes (1,2) and therefore ANP values are correlated to intracardiac pressures (3), it is well conceivable that diseases in which the intravascular volume is not enlarged, but yet intracardiac pressures rise, ANP release is enhanced. One such a disease is pulmonary embolism. Due to obstruction of (part of) the pulmonary blood flow, pressures on the right side of the heart are elevated thereby causing ANP concentrations to increase. With dissolution of the embolus these pressures decrease and concomitantly ANP levels would normalize. This hypothesis was tested and the results are described in chapter 5.1. In chronic myocardial infarction the intravascular volume is increased due to the remaining cardiac dysfunction with resulting activation of counter-regulatory mechanisms. At this stage, elevated ANP levels have been measured (4,5). However, at onset of infarction no enlargement of the intravascular volume is present. Due to the instantaneous onset of infarction, one might expect a sudden increase in intracardiac pressures (6) and thereby an increase in ANP concentrations, which increase is related to the degree of myocardial dysfunction. Therefore ANP concentrations were measured longitudinally during two days in patients with acute myocardial infarction. The results are described in chapter 5.2.

Chapter 5.1

Reciprocal changes in ANP and PRA during treatment of pulmonary embolism

Introduction

Pulmonary embolism is characterized by acute obstruction of part of the pulmonary blood flow, which regularly causes an elevation of the pulmonary arterial pressure and the right atrial pressure (7,8). We hypothesized that during dissolution of the embolus atrial natriuretic peptide levels would decrease. Therefore ANP levels were followed in 12 patients with proven pulmonary embolism. They were treated with heparin initially and oral anticoagulants thereafter. In addition, five patients with peripheral venous thrombosis, receiving the same anticoagulant regime, were followed. As peripheral venous thrombosis is not associated with increased atrial pressures, no change in ANP levels in these patients was expected.

Besides ANP, plasma renin activity (PRA) and aldosterone were measured and the relations between the plasma concentrations of these three salt-regulatory hormones were studied.

Patients and methods

Patients

Pulmonary embolism was diagnosed when patients suffered from acute dyspnoea, had chest pain attached to the respiration and showed clear defects on the technetium Tc99 perfusion lung scan. Twelve patients (5 females, 7 males, mean age ± SD = 47 ± 21 years) fulfilled these criteria and were enrolled in the study group. Five patients (3 females, 2 males, aged 41 to 75 years) with venous thrombosis in pelvic or upper leg veins were diagnosed clinically and by occlusive impedance plethysmography. On admission to the hospital none of the patients were hypertensive or had any other sign of cardiac or renal failure. Blood samples were collected (EDTA-tubes on ice) from all patients (supine position) on admission (day 1, before institution of anticoagulant therapy) and, if possible, daily thereafter at 8 a.m. for up to 11 days. Each patient was observed 7 to 10 times. In 2 patients with pulmonary embolism and in 1 patient with

venous thrombosis the first sample was taken on day 2 of treatment. Treatment consisted in the administration of heparin by continuous intravenous infusion (25,000 I.U. daily) and oral anticoagulants (acenocoumarol or phenprocoumon) for seven days, and solely oral anticoagulants thereafter. All patients received a standardized hospital diet (containing 10 to 12 g NaCl/day).

Methods

ANP was measured using the extraction method with the S-32 antibody. Plasma renin activity was measured using the Phadebas Angiotensin I test (Pharmacia Diagnostics, Sweden), and plasma aldosterone was measured using a direct radioimmunoassay (9).

Statistical analysis

The individual data of all 17 patients were analyzed by using the two-sided Spearman rank correlation test. The correlation coefficients (c.c.) relating to the 12 patients with pulmonary embolism were normalized (10) to calculate the p-value and the 95 % confidence interval (95 % C.I., for 8 degrees of freedom). Significance was assessed at the $\alpha=0.05$ level.

Results

Although initial values of ANP varied widely, the subsidence of clinical signs and symptoms of pulmonary embolism was attended with consistent changes in plasma ANP and PRA concentrations. Figure V−1 illustrates the changes in mean ANP and PRA values, the former significantly falling ($p<0.01$, mean c.c. −0.68, 95 % C.I.: −0.30 to −1.0), the latter significantly rising ($p<0.01$, mean c.c. +0.68, 95 % C.I.: +0.10 to +1.0). In eight individual patients the fall in ANP values reached statistical significance at the $\alpha=0.05$ level. In six patients the rise in PRA values became significant at this level. Intra-individually, the decrease in ANP values was significantly related to the increase in PRA values ($p<0.01$, mean c.c. −0.51, 95 % C.I.: +0.193 to −1.0). In the five patients with peripheral venous thrombosis treatment-related changes could be detected neither in ANP nor in PRA values. Interestingly, plasma aldosterone did not show any consistent change during treatment neither in the one nor in the other group of patients (pulmonary embolism: mean c.c. +0.06, 95 % C.I.: −0.22 to +0.34).

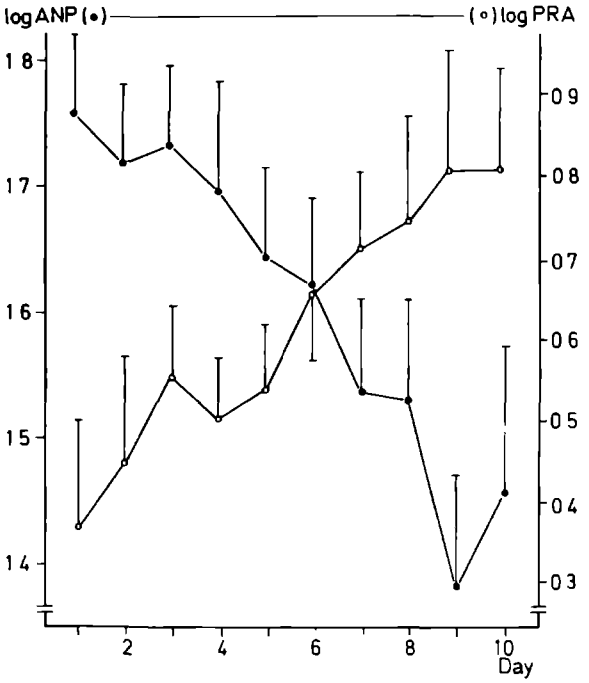

Figure V-1. Reciprocal changes in ANP (pg/ml) and PRA (nmol/l/h) levels (log scale) during treatment of pulmonary embolism. Bars denote mean ± SEM.

Discussion

Acute pulmonary embolism can increase the pressure on the right side of the heart (7,8,11,12) and thereby provide a stimulus to the secretion of ANP (3,13). We hypothesized that the dissolution of a pulmonary embolus will generally lead to restitution of normal pressures in the right atrium, and in consequence to normal values of ANP. In each patient with pulmonary embolism ANP decreased during anticoagulant treatment, in agreement with this hypothesis.

One might wonder whether anticoagulant therapy, mainly its change from heparin to oral anticoagulants may have a significant effect on ANP levels. Indeed, it has been observed in the rat that ANP effects are inhibited by heparin *in vivo* (14) whereas the plasma half life of (99-126)ANP is prolonged (15). If the change in ANP would be caused by heparin given to our patients during the first seven days of their treatment, an increase rather than the observed decrease in ANP levels in that period would be expected. Furthermore the absence of consistent ANP changes in the five patients with peripheral venous thrombosis

receiving the same anticoagulant regime strongly indicates that the observed ANP changes in pulmonary embolism are not related to the treatment regime.

The decrease in ANP values during treatment of patients with pulmonary embolism was attended with a proportional rise in PRA values. The somewhat surprising absence of consistent changes in plasma aldosterone indicates that on a dietary salt intake of about 10 gram sodium chloride sensible changes in sodium economy did not occur. The reciprocal changes in ANP and PRA in pulmonary embolism is another demonstration of the potency of the former peptide system, even in concentrations within the physiological range, to inhibit the activity of the renin angiotensin system (16,17). It remains to be elucidated if this inhibition occurs at the renal level. A more provocative explanation for the observed reciprocal changes would be that the intra-atrial pressure by itself may be a controlling factor of both ANP and PRA.

Chapter 5.2

ANP after myocardial infarction

Introduction

Immunoreactive atrial natriuretic peptide (ANP) concentrations are elevated in patients with congestive heart failure and correlate with intracardiac pressures (3,18). Little is known about changes in ANP concentrations after acute myocardial infarction (AMI). To determine whether there are any consistent changes, 38 patients with AMI who were treated succesfully were followed. Infarct size was assessed using the maximum creatine kinase value and left ventricular systolic function by gated blood pool scintigraphy in the acute (2 to 4 days) and the chronic phases (10 to 14 days) after infarction. In 24 patients who were catheterized, ANP was measured in the femoral vein, the femoral artery and the right ventricle and correlated to left ventricular pressures.

Patients and methods

Patients

Thirty-eight patients who were succesfully treated after acute myocardial infarction were studied. The patients (32 males, 6 females, mean age ± SD = 55.1 ± 9.1 years, range 38 to 70 years) were admitted to the hospital within 4.4 hours (mean ± SD = 2.3 ± 0.9 hours) after the onset of symptoms. After a diagnosis of acute myocardial infarction was established (based on the symptoms and the electrocardiogram of the patient), blood was drawn for determination of ANP and creatine kinase levels, and 30 E anistreplase (=APSAC, Beecham Pharmaceuticals Inc, Bristol, Tenn, USA) and 100 mg prednisone were given intravenously. Subsequently the patients were given heparin (25,000 I.U. daily) and treated conventionally, depending on the clinical conditions, using nitrates, ß−blocking agents, calcium antagonists, diuretics and antiarrhythmic drugs. It was not considered ethical by the local Hospital Ethical Committee to strictly protocol the conventional treatment. All patients received a low sodium diet (containing 3 to 4 gr NaCl/day). Blood for determination of ANP and creatine kinase was sampled (supine position) at the time of admission, and 3, 6, 9, 12, 15, 18, 24, 32, 40, and 48 hours later. The left ventricular systolic function (ejection fraction) was assessed using gated blood pool scintigraphy in the acute

phase (2 to 4 days after infarction) and in the chronic phase (10 to 14 days after infarction).

In 24 patients cardiac catheterization was performed 13.9 ± 5.5 days after admission. Blood was drawn from the femoral vein, the femoral artery, and the right ventricle. In addition, left ventricular systolic and end-diastolic pressures were monitored.

The location of the infarct (anterior vs. inferior) was determined by means of electrocardiography. Early reperfusion was assessed by electrocardiography, the occurrence of arrhythmias and the increase in time to the maximum creatine kinase value. In the 24 patients who were catheterized, the number of coronary vessels affected (one, two or three) was determined.

Methods

ANP was measured using the extraction method with the S−32 antibody. Addition of either anistreplase or heparin to plasma samples did not influence ANP values (results not shown).

Statistical analysis

The changes in ANP concentrations were studied by means of analysis of variance for repeated measurements followed by the paired t-test (Student). The influence of various factors (age, maximum creatine kinase value, infarct site, reperfusion, number of coronary vessels affected) on ANP concentrations were investigated by means of stepwise multiple-regression analysis. To establish any correlations between ANP values and other variables, the Spearman rank correlation test was used. Differences between ANP values from the femoral vein, the femoral artery, and the right ventricle sampled during cardiac catheterization were analyzed using the paired t-test (Student). Significance was assessed at the $\alpha=0.05$ level. All values are expressed as mean ± SD, unless indicated otherwise.

Results

During the study period of 48 hours after admission, all but four patients were classified as stage I of the Killip classification for congestive heart failure (19). The remaining four patients were classified as Killip stage II during the first and as Killip stage I during the second half of the study period. Because in these patients the individual mean ANP concentrations measured during the first 24 hours were not found to differ from the individual means of the concentrations

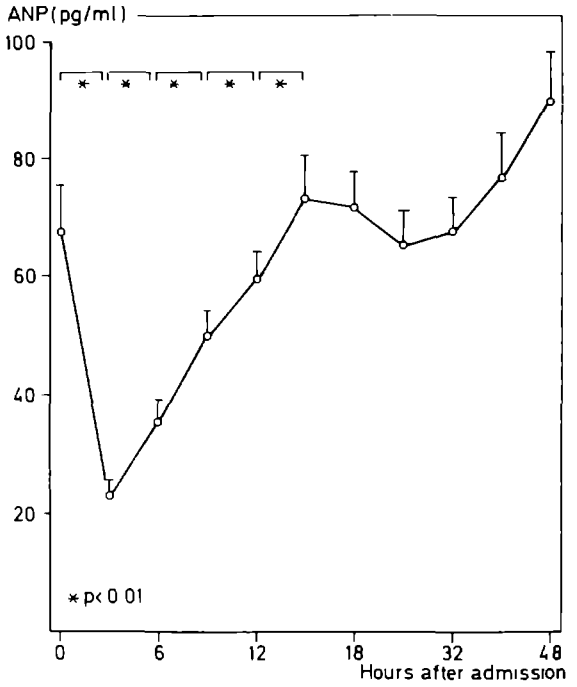

Figure V-2. Time course of ANP levels after admission to the hospital. Values are expressed as mean ± SEM.

measured in the other patients, the data of all 38 patients were analyzed as relating to one group.

Three hours after admission the mean ANP concentration (22.8 ± 17.8 pg/ml) was significantly lower than the mean ANP concentration at the time of admission (67.5 ± 44.5 pg/ml, $p<0.001$). Thereafter ANP levels increased steadily until 15 hours after admission (72.9 ± 43.1 pg/ml) (figure V−2). The mean ANP concentration 48 hours after admission (89.3 ± 51.1) was significantly higher than the mean ANP concentrations measured at the time of admission ($p = 0.04$) and 15 hours after admission ($p = 0.03$). Differentiation of the infarct as to size (small, maximum creatine kinase value < 1000 I.U./l, or large), as to site (anterior or inferior), and as to the occurrence of reperfusion did not influence the pattern of ANP concentrations.

The ANP concentrations measured at the time of admission to the hospital (ANP−1) were positively correlated with the infarct size (as determined by the maximum creatine kinase value, $r = 0.39$, $p<0.05$). The individual patient's

mean ANP concentration during the first 48 hours after infarction (i.m.ANP) was positively correlated with both the size of the infarct and the patient's age ($r = 0.36$, $p<0.05$ and $r = 0.43$, $p<0.01$ respectively). Neither the site of the infarct (anterior or inferior), nor the occurrence of reperfusion, nor the number of coronary vessels affected (one, two or three) influenced either the ANP−1 or the i.m.ANP level. Left ventricular systolic function, as assessed by gated blood pool scintigraphy, was negatively correlated with the i.m.ANP level in both the acute ($r = -0.46$, $p<0.05$) and the chronic phases ($r = -0.41$, $p<0.05$) after infarction. Only in the chronic phase (10 to 14 days, $r = -0.43$, $p<0.05$) did the ANP−1 level correlate with left ventricular function.

During cardiac catheterization the ANP concentrations in blood drawn at any of the three places mentioned did not correlate with left ventricular systolic pressure nor with left ventricular end-diastolic pressure (table V−1). As expected, ANP concentrations in blood drawn from the femoral vein were lower than in blood drawn from the femoral artery or the right ventricle. A close correlation was observed between ANP values measured in blood from these three places of origin (figure V−3).

Discussion

The atrial natriuretic peptide hormonal system can be regarded as acting antagonistically to the renin-angiotensin-aldosterone system, its main function

Table V-1. Comparison of ANP concentrations in blood sampled during cardiac catheterization (n = 24) with left ventricular systolic pressure (LVSP) and left ventricular end-diastolic pressure (LVEDP). No significant correlations were found.

	LVSP	LVEDP
femoral vein	0.19	0.09
femoral artery	0.08	0.27
right ventricle	0.15	0.32

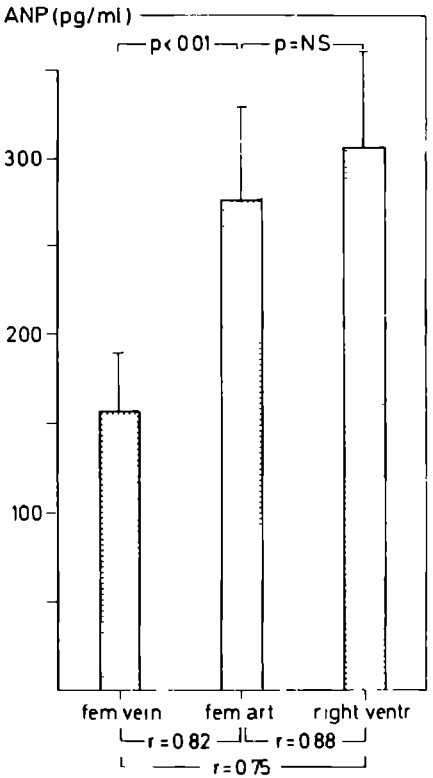

Figure V-3. ANP levels obtained during catheterization in the femoral vein, the femoral artery and the right ventricle and the correlations between these levels. Values are mean ± SEM.

being to protect the body from cardiovascular volume overload (20,21). In agreement with this view, high ANP levels have been observed in patients with congestive heart failure. ANP concentrations have been found to correlate with the severity of congestive heart failure and to decrease when the patients recovered (22-24). After myocardial infarction ANP values were found to correlate with maximum oxygen uptake during bicycle ergometry; this maximum is an index for the remaining ventricular function (25).

The systematic changes in ANP concentrations observed in this study confirm an assumption which Wencker *et al.* (26) made considering ANP values measured by direct assay in only four patients with acute myocardial infarction. Because plasma proteins interfere in the direct assay, prior extraction of plasma is recommended for accurate determination of ANP levels (27,28). In our study,

using an extraction method for measurement of ANP levels, an initial decrease followed by an increase in ANP levels was observed. We do share the explanation offered by Wencker *et al.* (26) of the observed phenomena. Immediately after the onset of infarction, intracardiac pressures rise causing a massive release of ANP up to depletion of atrial storage granules. Subsequently, ANP synthesis increases and plasma concentrations rise. On this hypothesis the ANP system, which protects the body from a volume overload, functions inadequately during the first few hours after acute myocardial infarction. Therefore, during these hours, the patient should be carefully monitored for any signs and symptoms of heart failure.

A different explanation of the changes in ANP concentrations might be that the initial surge of ANP is the result of release of ANP from ischaemic cardiac tissue. On this assumption higher ANP concentrations are to be expected in inferior than in anterior infarction, since in the former the atria are more often ischaemic than in the latter. However, results of multiple-regression analysis indicated that neither the site of the infarct (nor the occurrence of reperfusion) significantly influenced ANP levels at the time of admission (ANP−1) nor the individual mean ANP level during the first 48 hours (i.m.ANP), which is a measure of the release of ANP during this period.

In accordance with the hypothesis that release of ANP is determined by the severity of the infarction, both ANP−1 and i.m.ANP levels appeared to correlate with the maximum creatine kinase value, which is an index of the severity of the infarction, and with left ventricular function as assessed by gated blood pool scintigraphy. Individual mean ANP levels also correlated with patient age. As ANP values rise with advancing age (chapter 4.1), the observed correlation between the i.m.ANP level and the age of the patient indicates that after myocardial infarction the i.m.ANP level is a measure of ANP production rather than of ANP release due to ischaemic cardiac tissue.

Interestingly, although individual ANP values varied widely, the mean ANP levels ranged from 23 to 89 pg/ml. These values are considered to be in the normal range with the measurement method used (chapter 2.1). The absence of increased mean ANP levels in the patients without congestive heart failure is in agreement with observations of other investigators, who reported that mean ANP levels are not above normal in patients classified as class I according to the New York Heart Association (NYHA) Functional Classification (22,24,29).

Phillips *et al.* (30) reported that the mean ANP level in patients with acute myocardial infarction treated with streptokinase was not elevated as compared with the mean ANP level of patients treated without streptokinase. These investigators attributed this finding to the beneficial effect of streptokinase in

preserving the myocardium and myocardial function. The findings of normal mean ANP levels in our patients treated with anistreplase support and strengthen their view.

The weak but significant correlation between ANP values and cardiac function and the absence of elevated mean ANP levels in our study group of well recovered patients indicates that ANP may be useful to assess cardiac function only in cases of acute myocardial infarction complicated by overt congestive heart failure.

The absence of a significant correlation between ANP and left ventricular pressures during cardiac catheterization may come as a surprise. Cardiac catheterization was performed several days after infarction and at that moment all patients were classified as Killip stage I for congestive heart failure. Several investigators have reported a positive correlation between ANP values and intracardiac pressures (3,18,31). However, their observations were based on a wide range of intracardiac pressures. The absence of a correlation between ANP values and left ventricular pressures in our study group indicates that in these haemodynamically well-recovered patients, other factors may influence release of ANP and thereby obscure the relationship with intracardiac pressures.

In conclusion: ANP levels were studied after acute myocardial infarction. After the sudden onset of the infarct, ANP is released from atrial storage granules. Subsequently, depletion of these granules occurs causing a relative "hypo-ANP-ism". The weak relationships of infarct size and left ventricular function with, on the one hand, the ANP concentration on admission and, on the other hand, the individual mean ANP concentration during the first 48 hours after admission, mean that ANP can be of only limited value in assessing cardiac function after acute myocardial infarction.

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Part VI

SUMMARY AND CONCLUSIONS

The discovery by deBold and colleagues in 1981 (1) that the heart besides acting as a pump has an endocrine function roused world-wide interest. The newly-discovered hormone, called after its provenance, function and chemical structure atrial natriuretic peptide (ANP) can effect substantial diuresis, natriuresis and lowering of blood pressure. Within a few years' time its chemical structure was elucidated and its synthesis achieved, so that it became possible to develop methods for its assessment in plasma. A large number of publications on the atrial natriuretic peptide hormonal system have since appeared.

Part I of this thesis is a survey of the present state of knowledge about the novel hormonal system. The issues addressed in the present study can be summarized as follows:

- the evaluation of the reliability of a radioimmunoassay for ANP using in-house reagents including antisera and radioactive ligands,
- a study of material and physiological conditions which may influence the assessment of ANP concentrations,
- an analysis of the physiological relevance of the higher ANP values measured in aged subjects as compared to those measured in young subjects,
- an assessment of the involvement of atrial natriuretic peptide in pulmonary embolism and myocardial infarction

In Part II the results of the studies performed in connection with the first issue are presented. To establish a method for the measurement of ANP in plasma two sheep were immunized with synthetic (99-126)hANP. Thus two antibodies specific for the non-oxidized form of (99-126)hANP were obtained. Comparison with a commercially available antibody turned out in favour of the first-mentioned two antibodies. The results obtained with these three antibodies correlated well. With the two home-made antibodies two methods for measuring ANP concentrations were developed, a direct method and a method requiring prior extraction of plasma on Seppak C-18 columns. Although the direct method is considerably less laborious than the extraction method, its inaccuracy especially in the lower range of ANP concentrations, disqualifies it for reliable measurement of ANP values.

Using high pressure liquid chromatography (HPLC) analysis circulating forms of immunoreactive ANP in human plasma were studied. Both in normal subjects

and in patients with congestive heart failure three ANP immunoreactive forms were found. The main circulating form corresponds to (99-126)hANP. Whether the other two immunoreactive forms represent precursor or degradation products of (99-126)hANP and whether they are of physiological relevance remains to be elucidated. Stimulation of ANP release by ergometric bicycle exercise or volume loading predominantly stimulates the release of (99-126)hANP. This is an indication that this molecular form is the most important circulating ANP immunoreactive form in human blood (2,3). Cyclic guanosine monophosphate (cGMP) functions as the second messenger for ANP. Therefore cGMP values in plasma or urine reflect the biological effectiveness of ANP (4,5). A method for measuring cGMP concentrations in plasma is described in chapter 2.5. Combined assessment of ANP and cGMP concentrations is an effective way to study both the release and the effect of the atrial natriuretic peptide hormonal system.

In Part III a number of studies on the effects of various factors which may influence ANP values found in blood obtained from normal subjects are described. A study of different blood sampling procedures revealed that sampling in EDTA-tubes kept on ice and centrifuged within one hour gives the most reliable results. Platelets contain receptors for ANP and according to Richards *et al.* (6) their number in plasma can therefore influence the ANP concentration. The application of different modes of centrifugation, which yielded different amounts of platelets, did not, however, significantly influence ANP values. As storage of plasma at −20°C as well as repeated thawing decreases ANP immunoreactivity, it is recommended to store plasma at −80°C and prevent it from thawing more than once.

Neither venepuncture stress nor the phase of the menstrual cycle influence ANP values. ANP concentrations are higher in the early morning. The ensuing decrease in ANP may be explained by the decrease in venous return due to pooling of blood in the lower extremities. Like many other hormones, ANP concentrations are subject to endogenous variations. These variations cannot be entirely attributed to assay variations and therefore can stand in the way of a correct interpretation of the values found.

As the secretion of ANP is dependent on venous return to the heart, its values are higher in the supine than in the upright position. Ergometric bicycle exercise stimulates cardiac ANP secretion, which is of physiological significance because plasma levels of cGMP, the second messenger for ANP, rise proportionately. Using the extraction method reference values for ANP were determined under standardized conditions in 66 healthy volunteers recruited from the general population. In these subjects ANP concentrations ranged from 8.3 to 86.8 pg/ml

(mean ± SD = 35.3 ± 16.2 pg/ml). A positive correlation between the ANP value and age was found.

In Part IV a few studies dealing with age-related change in ANP concentrations are described. A study of the kinetic parameters of ANP revealed a similar plasma half-life but a markedly decreased total body clearance and consequently a decreased distribution volume of ANP in elderly subjects as compared to young subjects. This decreased distribution volume may contribute to the higher ANP values found in the elderly. The correlation observed in the elderly between ANP clearance and endogenous creatinine clearance, a measure for the number of functioning nephrons, is an indication of the important role of the kidney in ANP clearance. The higher ANP values in the elderly do not seem to be attributable to different circulating forms of ANP, as by means of HPLC analysis similar ANP immunoreactive profiles were observed in one young subject and one aged subject.

Dehydration induced by restriction of fluid intake to 25 % of normal did not cause significant changes in ANP levels either in young or in aged subjects. In contrast, volume loading (750 ml 0.9 % NaCl in 60 minutes) caused a pronounced increase in ANP values in the aged whereas no change occurred in the young. Although basal ANP values were higher in the elderly, basal plasma cGMP values were similar and rose to a comparable extent during volume loading. These findings point to decreased responsiveness of the ANP target-organs with advancing age. This hypothesis is supported by the results described in chapter 4.5.

Infusion of (99-126)hANP resulted in far higher concentrations of ANP in the elderly than in the young. On the other hand, basal plasma cGMP values, reflecting the biological effectiveness of ANP, were similar, and during low dose infusion (0.25 μg/min ANP) cGMP values rose to a comparable extent in both groups. At high dose infusion (2.0 μg/min ANP), cGMP rose more in the elderly. It may well be that this greater rise is due to the higher ANP concentrations reached during infusion in this group.

Low dose infusion did not result in significant haemodynamic effects in either group. A reduction in blood volume, as reflected in the haematocrit values, was observed in the young. Blood pressure fell and both heart rate and forearm blood flow rose at high dose ANP infusion, which is consistent with reports from other investigators (7–9). The effects were more pronounced in the elderly, which is also reflected in the greater rise in cGMP values.

Low dose ANP infusion did not produce consistent changes in plasma renin activity (PRA), aldosterone, cortisol, adrenaline or noradrenaline levels in either

group. During high dose ANP infusion a relative increase in PRA in both groups was observed, while aldosterone decreased only in the aged. No effect of ANP on cortisol and adrenaline was observed in either group. The lack of sympathetic stimulation as reflected in the noradrenaline values during infusion of the high, blood pressure lowering, dose of ANP is an indication that ANP modulates the sympathetic nervous system (10).

In general, the effects of high dose ANP infusion were more pronounced in the elderly. During infusion at this dose the ANP concentrations were, however, far higher in this group, which may explain the stronger effects. This is also expressed in the higher cGMP values that were reached in this group at high dose ANP infusion.

In view of

- the similar increase in cGMP in both groups during volume loading in contrast with a rise in ANP occurring only in the elderly,
- the similar basal cGMP values despite the higher basal ANP concentrations in the elderly,
- the comparable increase in cGMP during low dose ANP infusion despite the higher ANP concentrations reached in the elderly, and
- the fact that haemoconcentration - a measure for volume contraction - at low dose infusion was found only in the young,

it is plausible that with advancing age the responsiveness of the ANP target organs diminishes. This diminished responsiveness is compensated by a stronger response of the ANP hormonal system both to stimulation (e.g. by volume loading) and to infusion of exogenous ANP. Thus the system is capable, in the young and the old, to respond with similar potency to volume changes. At least part of this stronger response of the ANP hormonal system can be ascribed to a decreased clearance of ANP in the elderly.

In Part V two studies are described in which ANP concentrations were measured serially in patients with pulmonary embolism or myocardial infarction. In pulmonary embolism the obstruction of the pulmonary blood flow causes pressures in the right side of the heart to rise. As ANP values are correlated to atrial pressures, one may expect initially elevated ANP concentrations, which will fall back to normal as the pulmonary embolus dissolves under anticoagulant medication. Although in a group of twelve patients with pulmonary embolism the mean ANP concentration on admission was not elevated, it did decrease during anticoagulant therapy. Concomitantly, plasma renin activity increased. These phenomena were absent in a control group consisting of patients with venous thrombosis. These findings support the hypothesis that pulmonary embolism

causes a relative increase in ANP concentrations.

To follow ANP concentrations after myocardial infarction, ANP was measured during 48 hours in 38 patients with acute myocardial infarction. The concentrations decreased initially, but rose subsequently till 15 hours after admission. The initial ANP concentrations on admission as well as the individual mean ANP concentrations during the first 48 hours after admission correlated weakly, though significantly, with the size of the infarct and the left ventricular function. Neither the site of the infarct nor the occurrence of reperfusion nor the number of coronary vessels affected influenced the ANP concentration. The observed changes in ANP concentrations after infarction may be explained as follows. Acute myocardial dysfunction after infarction, by raising atrial pressures, causes a release of ANP from atrial storage granules. The ensuing decrease in ANP concentrations is attributable to depletion of these storage granules. At this stage, circulating ANP concentrations will decrease despite increased intracardiac pressures. Subsequently ANP is produced at greater rate causing its concentrations in the blood to rise again. The weak correlations of infarct size and left ventricular function with, on the one hand, the ANP concentration on admission and, on the other hand, the individual mean ANP concentration during the first 48 hours after admission, mean that ANP can be of only limited value to assess cardiac function after acute myocardial infarction.

To conclude: in this thesis the evaluation of a sensitive and reliable method for the measurement of ANP concentrations in humans is described. In addition, a number of factors to which attention should be paid when interpreting ANP values were studied. The ANP measurement method developed was used to study the physiological significance of the higher ANP levels observed in elderly subjects. Determination of ANP concentrations can be of value in studying certain pathophysiological phenomena associated with cardiovascular diseases, such as pulmonary embolism and acute myocardial infarction.

SAMENVATTING EN CONCLUSIES

De ontdekking in 1981 door deBold en collega's (1) dat het hart naast een pompfunctie tevens een endocriene functie bezit, wekte wereldwijde interesse op. Het nieuw ontdekte hormoon, naar zijn plaats van productie, functie en chemische structuur, het atrieel natriuretisch peptide (ANP) genoemd, kan een forse diurese, natriurese en bloeddrukdaling teweegbrengen. Binnen een paar jaar tijd werd de chemische structuur ontrafeld en het hormoon gesynthetiseerd, waardoor de mogelijkheid ontstond om een bepalingsmethode voor dit hormoon te ontwikkelen. Sindsdien is een groot aantal publicaties over het atrieel natriuretisch peptide hormonaal systeem verschenen.

In deel I van dit proefschrift wordt een kort overzicht gegeven omtrent het huidige begrip over dit nieuwe hormoonsysteem. De studies beschreven in dit proefschrift kunnen als volgt worden samengevat:

- de evaluatie van een radioimmunoassay voor ANP opgezet met zelf-ontwikkelde reagentia inclusief antiserum en radioactief ligand
- het bestuderen van factoren en fysiologische condities die de meting van ANP waarden kunnen beïnvloeden
- het bestuderen van de fysiologische betekenis van de hogere ANP waarden gevonden bij oudere mensen in vergelijking met jongere mensen
- het bestuderen van de betrokkenheid van het atrieel natriuretisch peptide bij longembolie en myocardinfarct

In deel II van dit proefschrift worden de resultaten van de studies uitgevoerd naar aanleiding van de eerste vraagstelling, beschreven. Teneinde een methode voor meting van het ANP in plasma op te zetten, werden twee schapen geïmmuniseerd met synthetisch (99-126)hANP. Hiermee werden twee antilichamen verkregen die specifiek zijn voor de niet-geoxideerde vorm van het (99-126)hANP. Vergelijking met een commercieel verkrijgbaar antilichaam viel in het voordeel van de eerste twee genoemde antilichamen uit. De resultaten zoals verkregen met deze drie antilichamen correleerden onderling goed met elkaar. Met de twee opgewekte antilichamen werden twee methoden ontwikkeld om ANP concentraties te bepalen, namelijk een directe methode en een methode die gebaseerd is op voorafgaande extractie van het plasma op Seppak C−18 kolommen. Hoewel de directe methode veel minder bewerkelijk is dan de extractie methode, is door het gebrek aan betrouwbaarheid met name in het lagere gebied van ANP waarden, deze methode ongeschikt voor een betrouwbare meting van het ANP gehalte.

Gebruikmakend van hoge-druk vloeistofchromatografie (HPLC) werden de

circulerende vormen van het immunoreactief ANP in menselijk plasma bestudeerd. Zowel in plasma van normale personen als in plasma van patiënten met decompensatio cordis werden drie ANP-immunoreactieve vormen gevonden. De voornaamste vorm komt overeen met (99-126)hANP. Het dient nog nader onderzocht te worden of de andere twee ANP-immunoreactieve vormen, voorloper- of afbraak-produkten van (99-126)hANP zijn en of zij enige fysiologische betekenis hebben. Stimulatie van ANP secretie door inspanning (fiets-ergometrie) of door volume expansie stimuleerde voornamelijk de (99-126)hANP vorm. Dit kan erop wijzen dat deze vorm de meest belangrijke circulerende ANP-immunoreactieve vorm is bij de mens (2,3). Cyclisch guanosine monophosphate (cGMP) functioneert als de cellulaire boodschapper voor het ANP. Derhalve weerspiegelt de cGMP waarde in plasma of urine het biologische effect van ANP (4,5). In hoofdstuk 2.5 wordt een methode voor het meten van cGMP waarden in plasma beschreven. De gecombineerde bepaling van zowel ANP als cGMP is een doeltreffende manier om zowel de afgifte als het effect van het atrieel natriuretisch peptide hormonaal systeem te bestuderen.

In deel III worden verschillende studies beschreven met betrekking tot het effect van verschillende factoren die de ANP waarde in bloed van normale personen kunnen beïnvloeden. Studie van verschillende bloedafname-procedures wees uit dat bloedafname in EDTA-buizen op ijs en gecentrifugeerd binnen een uur de meest betrouwbare resultaten oplevert. Bloedplaatjes bevatten receptoren voor ANP en derhalve zou volgens Richards *et al.* (6) de hoeveelheid bloedplaatjes aanwezig in plasma de ANP concentratie kunnen beïnvloeden. Het gebruik van diverse centrifugatie-methoden die verschillende hoeveelheden bloedplaatjes in het plasma opleverden beïnvloedde de ANP waarde echter niet. Omdat zowel opslag van plasma bij −20°C als het herhaald ontdooien van plasma de ANP-immunoreactiviteit vermindert, wordt aanbevolen om plasma bij −80°C te bewaren en om meer dan eenmalig ontdooien van plasma te voorkomen.

Noch de stress tengevolge van het aanprikken van een bloedvat noch de fase van de menstruele cyclus beïnvloeden de ANP concentratie. In de vroege ochtend worden hogere ANP concentraties gemeten. De hiernavolgende daling in ANP waarden zou verklaard kunnen worden door een daling van de veneuze terugvloed naar het hart als gevolg van stase van bloed in de onderste extremiteiten. Zoals veel andere hormonen, zijn ook de concentraties van het ANP aan endogene variaties onderhevig. Deze variaties kunnen niet volledig toegeschreven worden aan de bepalings-methodiek en kunnen derhalve de interpretatie van ANP waarden bemoeilijken. Omdat de secretie van het ANP

afhankelijk is van de veneuze terugvloed naar het hart, worden hogere ANP waarden gemeten in de liggende positie in vergelijking met de waarden gemeten in de staande positie. Inspanning op een fietsergometer stimuleert de ANP secretie uit het hart, wat van fysiologische betekenis is omdat ook de spiegels van het cGMP, de cellulaire boodschapper van het ANP, parallel met het ANP stijgen. Gebruikmakend van de extractie-methode werden referentie-waarden voor het ANP bepaald bij 66 gezonde vrijwilligers uit de bevolking. In deze personen varieerden de ANP concentraties van 8,3 tot 86,8 pg/ml (gemiddelde en standaard-deviatie = 35,3 ± 16,2 pg/ml). In deze groep werd een positieve correlatie gevonden tussen de ANP waarde en de leeftijd.

In deel IV worden enkele studies beschreven met betrekking tot de leeftijdsafhankelijke verandering van het ANP gehalte in plasma. Bij bestudering van de kinetische parameters van het ANP bleek dat oude personen eenzelfde plasma halfwaardetijd maar een verminderde klaring en derhalve ook een verminderd distributie-volume van het ANP hebben in vergelijking met jonge personen. Dit verminderde distributie-volume zou kunnen bijdragen aan de hogere ANP waarden gevonden bij ouderen. De gevonden correlatie bij de ouderen tussen de ANP klaring en de endogene creatinine klaring, een maatstaf voor de hoeveelheid functionerende nephronen, onderstreept het belang van de nier bij de klaring van ANP. De hogere ANP waarde bij de oudere personen kan waarschijnlijk niet toegeschreven worden aan andere circulerende vormen van het ANP omdat, gebruikmakend van HPLC analyse, een soortgelijk ANP-immunoreactief profiel werd gevonden bij een jonge en bij een oude persoon.

Dehydratie door restrictie van de vloeistof-inname tot 25 % van normaal veroorzaakte geen significante verandering in de ANP waarde noch bij jonge noch bij oude personen. Volume expansie (750 ml 0,9 % NaCl in 60 minuten) daarentegen veroorzaakte een forse stijging van de ANP waarde bij de ouderen terwijl geen verandering werd gevonden bij de jonge personen. Hoewel de basale ANP concentraties hoger waren in de oude personen, waren de plasma cGMP waarden hetzelfde en het cGMP steeg in gelijke mate gedurende de volume expansie. Deze bevindingen duiden op een verminderde respons van de doelwitorganen voor het ANP met het vorderen van de leeftijd. Deze hypothese wordt ondersteund door de resultaten beschreven in hoofdstuk 4.5.

Infusie van (99-126)hANP resulteerde in veel hogere ANP concentraties in de ouderen in vergelijking met de jonge personen. De plasma cGMP waarden, een weerspiegeling van het biologische effect van ANP, waren basaal hetzelfde, en stegen evenveel in beide groepen bij een lage dosering ANP (0,25 μg/min ANP). Bij hoge dosering ANP (2,0 μg/min ANP) steeg het cGMP meer in de oudere

groep. Het is heel goed mogelijk dat deze sterkere stijging veroorzaakt wordt door de hogere ANP concentraties die in deze groep tijdens infusie bereikt werden.

Lage dosering ANP veroorzaakte geen significante hemodynamische veranderingen in beide groepen. Bij de jongeren verminderde het circulerend volume, zoals weerspiegeld in de hematocriet-waarden. Bij de hoge dosering ANP daalde de bloeddruk en stegen zowel de hartslag als de bloeddoorstroming in de onderarm. Deze resultaten komen overeen met de resultaten zoals gevonden door andere onderzoekers (7-9). De effecten waren meer uitgesproken bij de ouderen, zoals tot uitdrukking komt in de sterkere stijging van cGMP waarden.

Lage dosis ANP veroorzaakte geen consistente veranderingen in plasma renine activiteit (PRA), aldosteron, cortisol, adrenaline of noradrenaline in beide groepen. Gedurende hoge dosis ANP werd een relatieve stijging van de PRA in beide groepen gevonden terwijl het aldosteron alleen bij de ouderen daalde. In geen van beide groepen beïnvloedde het ANP de cortisol of adrenaline waarden. Het gebrek aan activatie van het sympatisch zenuwstelsel zoals weerspiegeld in de noradrenaline waarden gedurende infusie van de hoge, bloeddrukverlagende dosis ANP is een aanwijzing dat het ANP het sympatische zenuwstelsel kan beïnvloeden (10).

In het algemeen waren de effecten tijdens infusie van de hoge dosis meer uitgesproken bij de ouderen. Gedurende infusie van deze dosis waren de ANP concentraties echter veel hoger in deze groep, wat de sterkere effecten kan verklaren. Dit wordt ook weergegeven in de hogere cGMP waarden bereikt in deze groep bij de hoge dosis ANP.

Gezien de:

- gelijke stijging van het cGMP in beide groepen in vergelijking met de ANP stijging alleen bij de ouderen gedurende volume expansie
- dezelfde basale cGMP concentraties ondanks de hogere basale ANP concentraties bij de ouderen
- vergelijkbare stijging van het cGMP gedurende infusie van lage dosis ANP ondanks de hogere ANP concentraties bij de ouderen
- hemoconcentratie, wat duidt op volume contractie, bij lage dosis ANP wat alleen bij de jongeren werd gevonden

wordt een verminderde gevoeligheid van de doelwit-organen voor ANP met het vorderen van de leeftijd aannemelijk gemaakt. Deze verminderde gevoeligheid wordt echter gecompenseerd door een sterkere respons van het ANP hormonale systeem, zowel op stimulatie (b.v. door volume expansie) als op infusie van exogeen ANP. Hierdoor is dit hormonaal systeem in staat om zowel bij jongeren als bij ouderen met eenzelfde capaciteit te reageren op volume veranderingen. Ten-

minste een gedeelte van de sterkere respons van het ANP hormonaal systeem kan toegeschreven worden aan de verminderde klaring van het ANP bij oude personen.

In deel V worden twee studies beschreven waarbij ANP concentraties opeenvolgend werden gemeten in patiënten met een longembolie of met een myocardinfarct. Door de obstructie van de longdoorbloeding bij longembolie stijgen de drukken aan de rechterkant van het hart. Daar ANP concentraties gecorreleerd zijn met de atriële drukken, verwacht men aanvankelijk verhoogde ANP concentraties die dalen wanneer de longembolus wordt opgelost door antistollingstherapie. Hoewel de gemiddelde ANP spiegel bij opname in een groep van twaalf patiënten met longembolie niet verhoogd was, daalde het ANP gedurende antistollings-therapie. Tegelijkertijd steeg de plasma renine activiteit. Deze bevindingen werden niet gevonden in een groep patiënten met veneuze thrombose. Dit ondersteunt de hypothese dat longembolie een relatieve stijging van de ANP concentraties veroorzaakt.

Om het verloop van ANP concentraties na een myocardinfarct te bestuderen, werd ANP gemeten gedurende 48 uur bij 38 patiënten met een acuut myocardinfarct. Het ANP daalde aanvankelijk, maar steeg daarna tot 15 uur na opname in het ziekenhuis. Zowel de individuele ANP concentratie gemeten bij opname als de individuele gemiddelde ANP waarde gedurende de eerste 48 uur na opname, correleerden met de grootte van het infarct en met de linker-ventrikelfunctie. Noch de plaats van het infarct noch het optreden van reperfusie noch het aantal coronairvaten getroffen, beïnvloedden de ANP concentraties. De waargenomen veranderingen in ANP concentraties na een infarct kunnen als volgt worden verklaard. Na een infarct treedt er een acute dysfunctie van het hart op die, door verhoging van de atriële drukken, een afgifte van het ANP uit de atriële granula teweegbrengt. De hierop volgende daling van het ANP kan toegeschreven worden aan een uitputting van deze granula. In deze fase zullen de ANP concentraties in het circulerende bloed dalen ondanks de verhoogde drukken in het hart. Vervolgens wordt het ANP met een grotere snelheid aangemaakt waardoor de concentraties in het bloed weer zullen stijgen. De zwakke correlaties tussen de grootte van het infarct en de linker-ventrikel-functie aan de ene kant en de ANP waarde bij opname en de gemiddelde ANP waarde gedurende de eerste 48 uur aan de andere kant, duiden erop dat het ANP slechts van beperkte waarde kan zijn om de hartfunctie na een myocardinfarct te bepalen.

Samenvattend: in dit proefschrift wordt een gevoelige en betrouwbare methode

om ANP concentraties bij mensen te bepalen, geëvalueerd. Tevens worden enkele factoren beschreven waarop gelet dient te worden wanneer men ANP waarden interpreteert. De meetmethode voor ANP werd gebruikt om de fysiologische betekenis van de hogere ANP waarden bij oudere personen te bestuderen. Bepaling van ANP concentraties kan van waarde zijn bij het bestuderen van bepaalde pathofysiologische verschijnselen samenhangende met cardiovasculaire ziektebeelden zoals longembolie en myocardinfarct.

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Curriculum vitae

De schrijver van dit proefschrift werd op 28 juli 1963 geboren te Pontianak (Indonesië). Nadat hij in 1981 aan het Canisius College te Nijmegen het VWO-diploma had behaald, begon hij met de studie geneeskunde aan de Katholieke Universiteit te Nijmegen. In juni 1983 behaalde hij het kandidaatsexamen (cum laude) en in maart 1986 het doctoraalexamen. Van maart 1986 tot november 1986 was hij in het kader van zijn wetenschappelijke stage werkzaam op het laboratorium voor Endocrinologie, gedurende welke periode een bepalingsmethode voor het atrieel natriuretisch peptide werd ontwikkeld. Het verslag hierover werd bekroond met de Universiteitsprijs 1987. Nadat hij het semi-arts examen in oktober 1987 met goed gevolg had afgelegd werkte hij bij de afdeling Experimentele en Chemische Endocrinologie (hoofd: Prof.Dr. Th.J. Benraad) en de afdeling Endocriene Ziekten (hoofd: Prof.Dr. P.W.C. Kloppenborg) aan de in dit proefschrift beschreven studies.

STELLINGEN

Behorende bij het proefschrift

ATRIAL NATRIURETIC PEPTIDE

In het openbaar te verdedigen op
dinsdag 12 december 1989
des namiddags te 1.30 uur
precies

door

Adriaan C.I.T.L. Tan

STELLINGEN

1. Het bestaan van zowel een cardiaal hormoon 'atrieel natriuretisch peptide', een (veronderstelde) neurotransmittor 'brein natriuretisch peptide' en een paracrien hormoon 'urodilatine' wijst op de aanwezigheid van een 'natriuretisch peptiderg systeem' in de regulatie van de water- en zoutbalans.

2 In menselijk bloed circuleren naast (99–126)hANP ten minste nog twee andere ANP-immunoreactieve stoffen.

dit proefschrift

3. De procedure van afname en behandeling van bloed is van wezenlijke invloed op het immunoreactieve ANP-gehalte.

dit proefschrift

4. De endogene ANP-spiegel kan intra-individuele variaties vertonen die niet volledig verklaard kunnen worden door intra-assay-variaties

dit proefschrift

5 De hogere ANP spiegels in het bloed van oudere mensen is tenminste voor een deel toe te schrijven aan het verminderde verdelingsvolume bij deze personen.

dit proefschrift

6. Met toenemende leeftijd vermindert de gevoeligheid van de doelwit-organen voor ANP. Deze verminderde gevoeligheid wordt echter gecompenseerd door een sterkere stijging van de ANP-spiegel als reactie op zowel endogene als exogene stimuli.

dit proefschrift

7 De daling van de ANP-spiegel na een acuut myocardinfarct wordt veroorzaakt door een depletie van de atriele granula.

dit proefschrift

8 Zowel de concentratie van ACTH als de concentratie van cortisol in bloed stijgt met het vorderen van de leeftijd.

eigen waarneming

9. Wanneer men het 'oproepsein' van de arts beschouwt als een draagbaar telefoonapparaat, wordt het de hoogste tijd een draagbaar automatisch antwoordapparaat te ontwerpen.

10 Wanneer de huidige tendens zich voortzet kan de term 'openbaar vervoer' beter worden vervangen door 'verplicht vervoer'.

11. Gezien de praktijk in het ziekenhuis zou de benaming 'co-assistent' dienen te worden vervangen door 'sub-assistent'

12. De term 'voetbal-vandalisme' kan beter worden vervangen door 'stadion-oorlog'.

13 De uitdrukking 'hagelstenen zo groot als duive-eieren' zegt meer over de grootte van hagelstenen dan over de grootte van duive-eieren

14. In tegenstelling tot 'post-partum depressie' kan aan het bestaan van een 'post-promotie depressie' ernstig getwijfeld worden

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