ATRIAL NATRIURETIC PEPTIDE AND STREPTOZOTOCIN-INDUCED DIABETES IN RATS

by

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(ABSTRACT)

This study was undertaken to determine whether immunoreactive atrial natriuretic peptide (irANP) concentrations in plasma and atrial tissue are altered in experimental diabetes mellitus (DM), and to compare the response of the DM and normal groups to exogenous administration of ANP. DM was induced by intraperitoneal injection of 45 mg/kg streptozotocin in male Sprague-Dawley rats. After three weeks of established DM (glucosuria and blood glucose > 250 mg/dl), plasma irANP levels were 149.6 $\pm$ 19.4 pg/ml in the DM group (n = 18) and 86.3 $\pm$ 12.9 pg/ml in the normal group (n = 12, $p < 0.01$). Atrial tissue irANP levels were significantly lower in the DM group (38.1 $\pm$ 7.8 ng/mg, n = 7) than in the normal group (60.1 $\pm$ 1.3 ng/mg, n = 4, $p < 0.02$). In response to intravenous infusion of ANP (2.5 ug/kg prime, followed by 0.1 ug/kg/min for 30 minutes), urine flow rate and urine sodium and potassium excretion rates increased significantly in the normal group (n = 6, $p < 0.05$), while no significant responses were found in the DM group (n = 6). It is concluded that plasma levels of ANP are significantly elevated in streptozotocin-induced diabetes in rats, and that atrial tissue stores are significantly depleted in this diabetic model. In addition, the renal response to exogenously administered ANP appears to be diminished in streptozotocin-induced DM.

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CHAPTER I
INTRODUCTION

Atrial natriuretic peptide (ANP) is a polypeptide hormone synthesized, stored, and released primarily by mammalian atrial cardiocytes. It has potent diuretic, natriuretic, and hypotensive actions, and it is believed to play a significant role in regulating sodium balance and blood pressure (38). It affects major blood pressure control points, including renal sodium and water excretion, vessel tonicity, aldosterone production, heart rate, cardiac output, renin secretion, and arginine vasopressin release (38). Plasma ANP levels increase in response to acute and chronic plasma volume expansion and are increased in disease conditions such as congestive heart failure and renal insufficiency. These findings suggest that ANP is important in body fluid homeostasis (12). Diabetes mellitus is a complex metabolic disorder which is accompanied by dysfunction of the cardiovascular, endocrine, and renal systems, and derangement of body fluid homeostasis mechanisms (75).

A. PRINCIPLE AIMS

Many of the body systems affected by diabetes mellitus are closely associated with systems in which ANP is also involved; however, little is known about physiologic changes of ANP in diabetes. This investigation was designed to compare plasma and atrial tissue immunoreactive ANP (irANP) concentrations in normal rats with concentrations in streptozotocin-induced diabetic rats. In addition, the effects of exogenously administered ANP on urine flow rate and urine sodium and potassium excretion rates in normal and diabetic rats was studied.

B. LITERATURE REVIEW

1. Atrial Natriuretic Peptide

a. History

The discovery of atrial natriuretic peptide (ANP) was preceded by the observation of Kisch in 1956 (94) and Jamieson and Palade in 1964 (87) that mammalian atrial cardiocytes are morphologically differentiated as both contractile and secretory cells. The secretory nature of these cells, as compared to the ventricular cardiocytes, is expressed by a highly developed Golgi apparatus, the presence of a high proportion of rough endoplasmic reticulum, and membrane-bound storage granules, the specific atrial granules (38) (Figure 1). The granules tend to be larger and more numerous in small mammals as compared to larger mammals, with rats having up to 600 specific atrial granules per atrial cell with diameters ranging from 200-500nm (25). All mammalian species studied thus far have been shown to have specific atrial granules (flying squirrel, rat, mouse, guinea pig, cat, rabbit, dog, bat, hamster, pig, ox, and human), and granules are also found in the ventricles of nonmammalian vertebrates and in the heart of elasmobranchs (40).

The investigations of the atrial granules actually progressed through three stages. First, Berger and Bencosme demonstrated that the specific atrial granules were not counterparts of known organelles, such as lysosomes or microbodies (16), then deBold and Bencosme proved that the granules do not contain catecholamines, as had been suggested by some investigators (44). Finally, histochemical investigations at the light microscopic level carried out by deBold and Bencosme (43, 46) suggested that the granules store a random coiled, basic polypeptide that is synthesized, stored, and

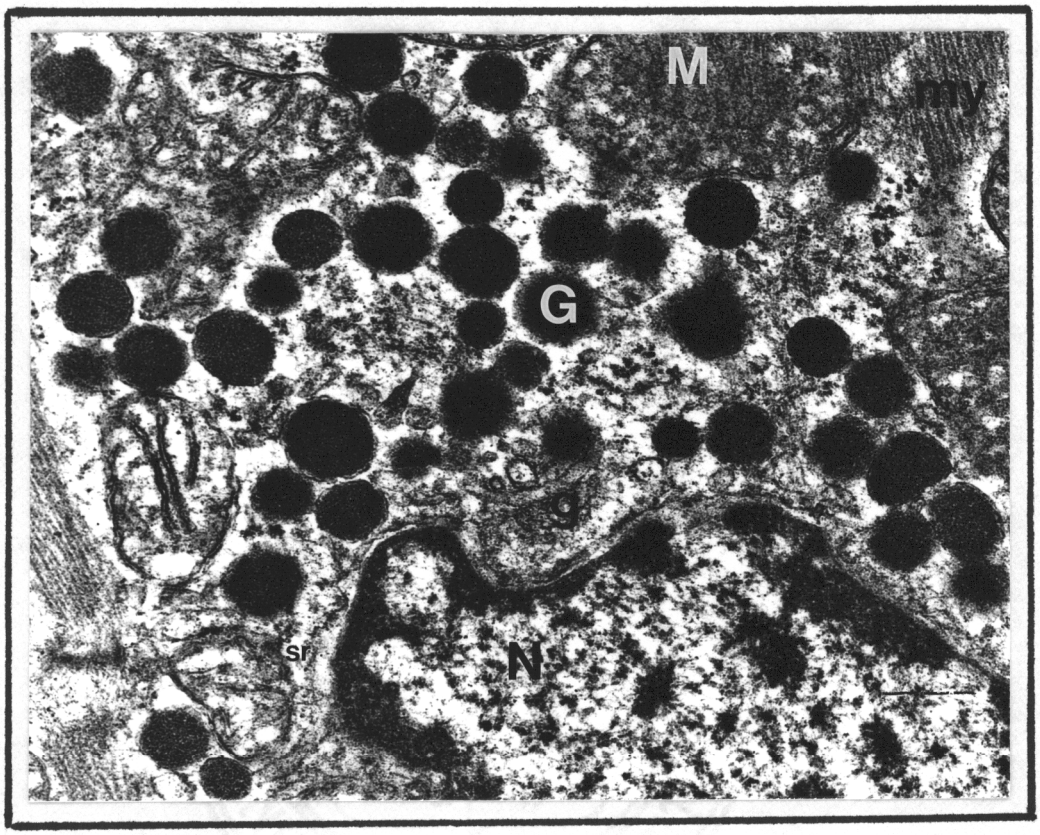

FIGURE 1: Electronmicrograph of atrial myocardium from normal rat. G = atrial specific granules, N = nucleus, g = Golgi apparatus, M = mitochondria, sr = sarcoplasmic reticulum, my = myofibril. Magnification x 27,500. Bar = 500nm.

released by atrial cardiocytes in a manner similar to that found for known secretory cells (42). These studies, combined with morphometric investigations wherein it was discovered that dietary changes in salt and water intake altered the granularity of rat atrial cardiocytes (39), led to the hypothesis that the atrial granules store a polypeptide involved in the regulation of fluid and electrolyte balance (38).

In 1981, deBold et al. (45) demonstrated that the supernatants of atrial myocardial homogenates cause a rapid and potent natriuresis, diuresis, and hypotensive response when injected intravenously into normal rats, while injection of ventricular myocardial extracts does not. They suggested that the substance in the rat atrial extracts be termed atrial natriuretic factor. This classic study touched off an intense interest in atrial natriuretic peptides by several groups in Japan and North America (25).

By using a tissue fractionation procedure, deBold's group was able to show that the atrial fraction with the highest natriuretic and diuretic activity was the fraction containing the granules (41). By 1985, immunochemical techniques using antisera raised against ANP peptides clearly localized the peptides within the atrial granules (27) (Table 1).

b. Structure and Processing

Over the past few years, several groups have independently isolated a series of closely related peptides that share virtually the same biological activity, and this has led to a rather confusing array of terms referring to atrial peptides (atrial natriuretic factor, atriopeptin, cardionatrin, auriculin, atrial natriuretic polypeptide, and atrin) (26). For the sake of simplicity, and also because it is no longer an unknown "factor," the term atrial natriuretic

TABLE 1: History of events leading to the characterization of ANP.

MAJOR EVENTS	PRINCIPLE INVESTIGATORS	YEAR	REFERENCE
1) Discovery of atrial specific granules	Kisch Jamieson and Palade	1956 1964	94 87
2) Cytochemical demonstration of unique nature of atrial specific granules	Berger and Bencosme	1971	16
3) Determination that atrial specific granules are not a storage site for catecholamines	deBold and Bencosme	1973	44
4) Histochemical demonstration that atrial specific granules contain a polypeptide	deBold	1978	46
5) Morphometric studies show that atrial granularity changes with different salt and water intakes	deBold	1979	39
6) Discovery that intravenous administration of atrial extracts results in natriuresis and diuresis	deBold	1981	45
7) Localization of natriuretic activity to atrial specific granules	deBold	1982	41
8) Determination of ANP amino acid sequence	Flynn	1983	56
9) Immunohistochemical localization of ANP to atrial specific granules	Chapeau	1985	27

peptide (ANP) will be used in this paper when referring to the 28-residue circulating form of the peptide.

A variety of natriuretic peptides of differing lengths have been isolated from rat and human atria, however, all share the same core structure with a 17-membered ring formed by a disulfide bond between Cys_7 and Cys_{23}, which is essential for biologic activity (12, 25, 26, 108). All fully active peptides have the C-terminal sequence -Phe-Arg-Tyr, while manipulation of the N-terminal extension does not significantly alter the bioactivity of the peptide, unless its length is greatly extended, which decreases activity (1, 12, 26,). It now seems likely that the variability of the isolated structures is largely artifactual, arising from different degrees of proteolysis during various isolation procedures (12, 26, 108).

This core group of natriuretic peptides constitutes the carboxyterminus of a 152 amino acid and 151 amino acid pre-pro-peptide in rats and humans, respectively (1, 12,). The sequence of the pre-pro-peptide was deduced from complementary DNA copies of ANP-specific mRNA (12), and it is divided into three regions: a signal peptide, connecting peptide, and ANP (1). Cleavage of the signal peptide gives rise to a 126 amino acid propeptide (proANP, gamma ANP), which is the predominant storage form of the ANP peptide (12, 26). The 28 amino acid carboxyterminus of the propeptide constitutes the active, circulating form of ANP (1, 12). This 28 amino acid form, often referred to as alpha ANP, is identical in the human and rat, except that Met_{12} in man is replaced by Ile in the rat (1) (Figure 2).

The method by which proANP is processed to produce the 28 amino acid ANP has received considerable attention. Generally, peptide hormones are stored in secretory granules where maturation takes place by serine proteases (150). However, in the case of ANP, the atria appear to contain predominantly, if

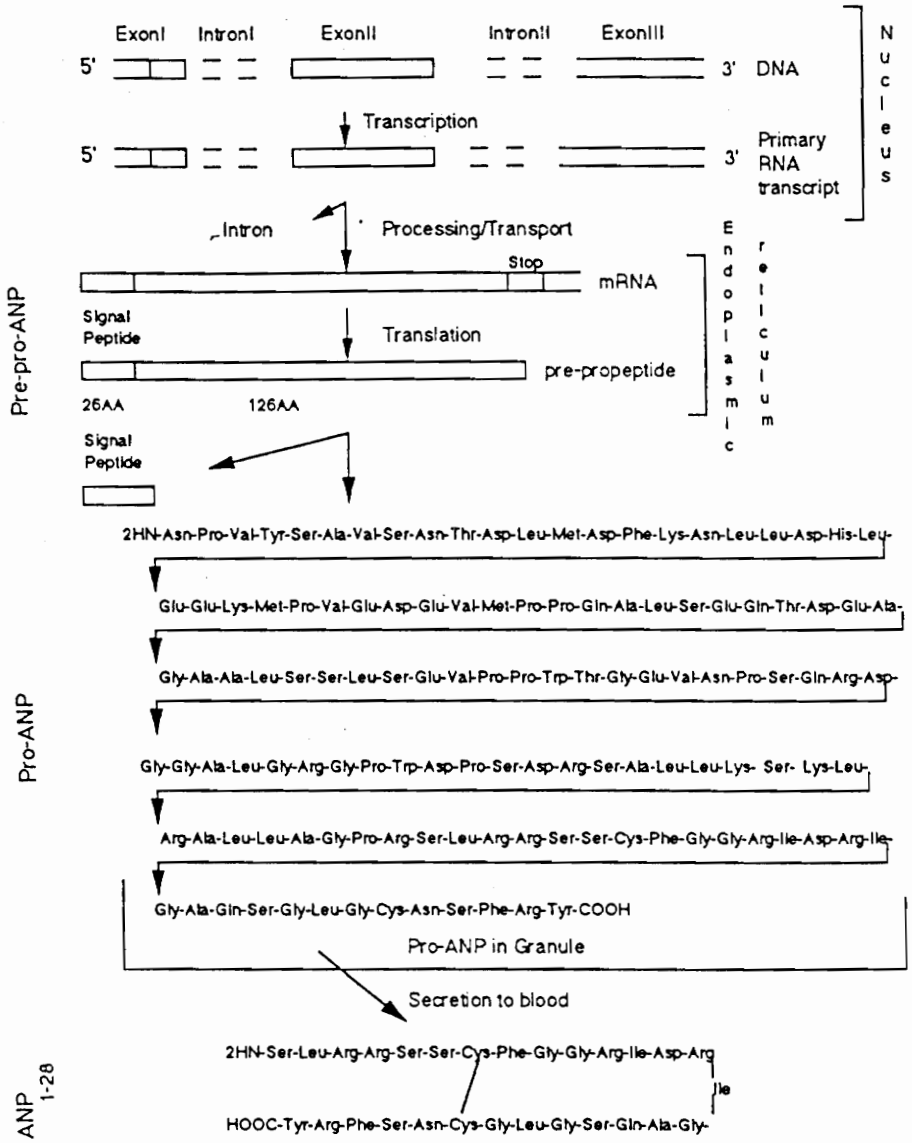

FIGURE 2: Synthesis and amino acid structure of rat pro-ANP and ANP. Adapted from Ballermann and Brenner (12) and Ackermann (1).

not only, the proANP form of the molecule. This suggested that the change of proANP takes place in the blood after secretion from the granules, but this theory was discounted because proANP cannot normally be detected, even in blood taken from the coronary sinus, and activation was shown to occur in isolated heart preparations perfused with media that did not contain serum (150). Another theory was that activation may be mediated by endothelial cells lining the atrium, but Imada's group disproved this possibility by demonstrating that proANP was not activated when treated with cultured endothelial cells (82).

This seems to leave only one other possibility, which is that the processing occurs at the time of secretion. This hypothesis was recently proven by Imada's group when they identified a specific activating enzyme for proANP in the atrium that selectively cleaves the Arg_{98}-Ser_{99} bond (82). This enzyme, which they called atrioactivase, is bound to the microsomal and plasma membrane fraction of atrial extract, which suggests that it works on proANP during the secretion process of atrial granules (82), which is thought to occur by exocytosis (118).

c. Tissue Distribution of ANP

ANP is synthesized primarily by atrial cardiocytes, but specific pre-pro-ANP mRNA has also been detected in cardiac ventricles, lungs, pituitary, aortic arch, and hypothalamus of the brain (104, 150) (Table 2). Within the heart, the right atrium contains about 2.5 times more ANP than the left atrium (25, 27, 84), and the ventricle contains about 100 times less pre-pro-ANP than the atria (104). The lungs also contain approximately 100 times less pre-pro-ANP than the atria, and the hypothalamus contains only trace amounts (104). In fetal rats, the cardiac ventricles contain approximately the same

TABLE 2: Tissue distribution of ANP.

ORGAN
Right atrium
Left atrium
Plasma
Ventricles
Lung
Aortic arch
Brain, hypothalamus
Brain, whole
Adrenal gland

amount of pre-pro-ANP as the atria, so the ANP gene appears to be actively transcribed in both the atria and ventricles of the fetus (62, 104). During development, the tissues diverge, such that the concentration of ANP in the atria increases with age, whereas it decreases in the ventricles (62). The ventricular ANP is not packaged in secretory granules, but appears to be secreted as it is formed (62, 104).

In these extra-atrial tissues, the storage forms of ANP are not exclusively proANP, but many processed forms occur, which suggests that the processing site in these tissue cells may be different from that of the atria (82).

The role of ANP in extra-atrial tissue is not known at present. In the brain, ANP is localized to neurons in regions involved in cardiovascular regulation, and in the aortic arch ANP distribution is similar to the distribution of aortic baroreceptors, so it may play a role in modulating these receptors (62). It is possible that ANP also serves as an autocrine factor with a local regulatory role in tissues such as the lung (62).

d. Mechanisms controlling release of ANP

There are numerous factors which may have an effect on the release of ANP from atrial cardiocytes, but the most effective and well-documented stimulus for ANP release is atrial distension (12, 26, 62, 65, 70, 72, 99). It had long been suspected that the atria contain pressure sensors that contribute to pressure and volume regulation. These suspicions were based on the findings of Henry et al. (76) that inflation of a balloon in the right atrium in the absence of volume expansion causes an increase in urine flow rate, and conversely, Goetz et al.'s (63) finding that acute volume expansion without atrial distension fails to produce diuresis. Ruskoaho's group was able to show that an increase in right

atrial pressure in isolated, perfused rat hearts causes the release of ANP into the perfusion fluid, thus proving that the phenomenon mentioned above is due at least in part to the release of ANP (132).

Right atrial pressure and distension can be increased by any factor that increases venous return, such as acute and chronic increased plasma volume, venoconstriction, supine posture, and immersion in water, which increases central blood volume, and elevated plasma ANP levels have been found to accompany these conditions (26, 97, 99, 100, 132). Plasma ANP levels have also been shown to increase with paroxysmal atrial tachyarrhythmias (12, 72, 132).

It has been shown that plasma ANP levels are affected by a number of circulating hormones (12, 26, 70, 108, 132). Intravenous administration of pressor doses of vasopressin, angiotensin II, or epinephrine result in dose dependent increases in plasma ANP in both rat and man, but it seems most likely that this is an indirect response secondary to the hemodynamic actions of these hormones, which cause a decrease in venous capacitance leading to increased right atrial pressure (70, 132). One hormone that does appear likely to have a direct stimulatory effect on ANP release is endothelin (139, 141). Endothelin is a recently discovered hormone secreted by endothelial cells, and it has been shown in primary atrial cell cultures to enhance ANP secretion (141). Ballermann et al. (11) also demonstrated that rat plasma ANP levels rise 12 hours after the initiation of deoxycorticosterone administration, which suggests that ANP is secreted in response to mineralocorticoid-induced volume expansion. They also found that this secretory response was accompanied by an increase in atrial pre-proANP mRNA activity, suggesting that ANP plays a role in the phenomenon of mineralocorticoid escape (11). It also seems that a paracrine factor secreted by intra-atrial blood vessels and/or the endocardium can inhibit

ANP release (137). This factor is thought to be similar to endothelium-derived relaxing factor (EDRF) because inhibition of EDRF by methylene blue, oxyhemoglobin, or hydroquinone, leads to an increase in basal, unstimulated ANP secretion. The fact that EDRF-inhibition resulted in increased ANP release without other stimuli also suggests that there is a continuous release of the factor that produces a tonic inhibition of ANP secretion (137).

In addition to the possible hormonal influences on ANP secretion mentioned above, there has been some speculation that the release of ANP from the heart may depend on the action of an ANP-releasing hormone from the anterior pituitary (54, 70, 170). Zamir et al. (170) demonstrated that both basal and volume-stimulated release of ANP are markedly reduced in rats which have undergone hypophysectomy, but that ANP levels are restored with reimplantation of the anterior pituitary under the kidney capsule. This suggests either that some as-yet unidentified hormone is released from the anterior pituitary which acts on the atrial myocyte to facilitate the release of ANP, or that a known pituitary hormone affects ANP release indirectly through its action on other endocrine organs such as the thyroid or adrenal cortex (170).

Considerable investigation has been devoted to determining whether the release of ANP is affected by neural pathways (26, 62, 70, 101, 128, 132). It has been shown that bilateral cervical vagotomy (101, 170) and beta blockade with propranolol (101) do not significantly affect ANP release stimulated by atrial stretch. In other studies it has been demonstrated that plasma ANP levels do not change during moderate sympathetic or vagal nerve stimulation unless atrial pressure is altered (128). The most convincing evidence that neural pathways are not involved in stretch-induced ANP release comes from studies involving isolated perfused hearts where marked increases in ANP in the perfusate are obtained in response to

increasing right atrial pressure (70, 132).

Though the findings mentioned above suggest that neuronal pathways are not necessary for stretch-induced ANP release, a role for autonomic nerves in the control of ANP release cannot be entirely excluded, as it has been shown that both epinephrine and acetylcholine stimulate ANP secretion in isolated perfused hearts (132). Phenylephrine and methoxamine, both alpha$_1$-receptor agonists, also stimulated ANP release, and the effect of epinephrine was attenuated by metoprolol, a beta-adrenoceptor antagonist, thus suggesting that both alpha$_1$ and beta adrenoceptors mediate the effect of epinephrine on ANP's release (132). In addition, the response to acetylcholine was blocked by atropine, suggesting that muscarinic receptors are involved in mediating acetylcholine's effect on ANP release (132). Ruskoaho et al. (132) point out that these findings indicate that the nervous system may have a modulatory role in ANP secretion, despite the findings that intact cardiac innervation is not necessary for stretch-induced ANP secretion. Further study is needed to clarify this proposed modulatory role of cholinergic and adrenergic substances on ANP release. There is little doubt that the autonomic nervous system indirectly affects ANP release, as alterations in sympathetic nerve activity change venous and arterial tone, and thus atrial pressure (70).

Another factor that has been examined as having a possible role in ANP release is plasma sodium concentration. There are numerous reports that high sodium intake is associated with increased plasma ANP levels (39, 133, 149), but the most likely explanation for this finding is that high sodium intake osmotically induces plasma volume expansion, which in turn increases atrial pressure and stimulates ANP release (7). Arjamaa et al. (7) bathed isolated atria in sodium chloride or hypertonic choline chloride and found that ANP release was increased, however, the changes in sodium chloride concentra-

tion and osmolality necessary to produce this response were very high and beyond the physiologic range (70). Nishida et al. (110) infused small volumes of highly concentrated sodium chloride solutions in dogs, and found that this rapid increase in plasma sodium concentration, which was within the physiological range, did not result in increased plasma ANP levels. They also concluded that high sodium intake-induced ANP secretion is due to the associated plasma volume expansion, rather than increased plasma sodium (110).

Atrial stretch is now generally agreed to be the primary stimulus of ANP release, but it is not clear how stretch actually induces this secretion. Two major cellular signal pathways have been reported to be involved in the secretion of hormones from endocrine glands (132). One pathway uses the second messenger, cyclic adenosine monophosphate (cAMP), while the other employs a combination of second messengers, including calcium ions, inositol triphosphate (IP_3), and diacylglycerol (DAG) (99, 132). IP_3 and DAG are both generated from membrane phosphoinositides, and each represents a branch of the phosphatidyl inositol system (132). Cytosolic calcium may be increased by entry into the cell from the extracellular fluid through voltage and receptor-operated channels, or it may be mobilized from intracellular calcium pools, such as the sarcoplasmic reticulum. The final step in these two pathways is phosphorylation of particular proteins which aid in the transport and fusion of secretory granules with the cell membrane (70). The protein phosphorylations are mediated by protein kinases. Protein kinase A is activated by cAMP, and protein kinase C is activated by DAG (132) (Figure 3).

With this background in mind, the contribution of each of these pathways to ANP secretion has been investigated by several groups by using pharmacological agents that mimic the actions of the various second messengers (70, 99, 132). The investigations were carried out by using isolated perfused rat

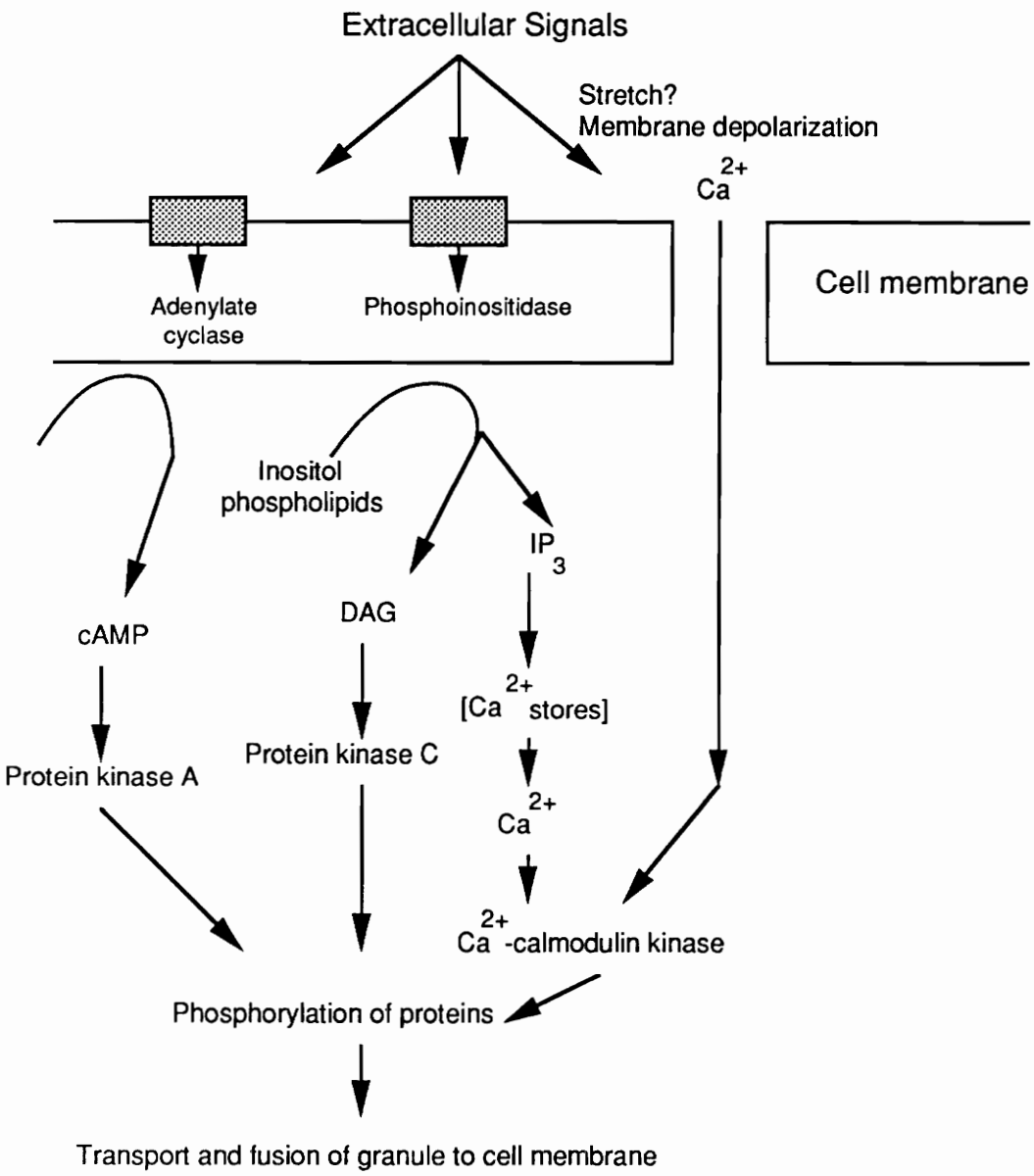

FIGURE 3: Proposed mechanisms for eliciting ANP release. Adapted from Ruskoaho (132). Shaded boxes represent receptor complexes.

hearts, adding various pharmacological second messengers, and measuring ANP levels in the perfusate. The action of DAG was mimicked with the phorbol ester 12-O-tetradeanoyl-13-acetate (TPA), which acts directly on protein kinase C. TPA produced a gradual increase in ANP secretion, whereas the biologically inactive phorbol ester, 4-alpha-phorbol-12, 13-didecanoate did not alter basal ANP secretion (70, 99, 132). This finding suggests that protein kinase C plays a role in ANP secretion from atrial cardiocytes.

Free calcium can be experimentally introduced into the cell with the ionophore A23187 or the calcium channel agonist Bay k8644 (70, 99, 132). When calcium and DAG are combined, they act synergistically to activate protein kinase C, and it was shown accordingly that the addition of A23187 or Bay k8644 plus TPA to the perfusion fluid resulted in a more than additive response in ANP secretion (70, 99, 132). These findings further support the idea that calcium-activated protein kinase C plays a role in ANP secretion. In addition, it was found that adding Bay k8644 or increasing concentrations of calcium in the perfusate without TPA also resulted in a marked increase in ANP secretion. This concept supports the theory that calcium is involved in ANP secretion (132). It should be noted, however, that deBold and deBold (47) recently reported that basal ANP release was increased by perfusion of isolated rat atria with calcium-free media. They noted the discrepancy with Ruskoaho's findings, but offered no explanation, and concluded that stretch-induced ANP release is an extracellular calcium-independent process. Similarly, Sei and Glembotski (141) reported that the basal rate of ANP release is unaffected by the concentration of calcium in the medium, but they found that endothelin- and phenylephrine-stimulated ANP release do require both intra- and extracellular calcium for maximal effect. Furthermore, these two stimuli of ANP release both stimulate IP_3 and DAG formation in isolated

hearts and primary heart cultures in the presence of extracellular calcium (141). These results suggest that basal ANP secretion may be a calcium-independent process, but that stimulated ANP secretion does require a source of calcium.

Forskolin is a compound which elevates cAMP levels in myocardial cells by activating adenylate cyclase, so forskolin was used to explore the role of cAMP-dependent protein kinase in ANP secretion. Forskolin alone was found to result in a relatively small increase in ANP release, but forskolin plus TPA produced a greater than additive increase in ANP release (99). It is well established that a cAMP-dependent protein kinase catalyses phosphorylation of sarcolemmal slow calcium channels in the heart, and these results show that events in the calcium-activated protein kinase C messenger system are closely related to those in the cAMP system in regulating ANP release (132).

In summary, these findings all suggest that cytosolic calcium is critical at least for stimulated ANP release and that the phosphatidyl-inositol-phosphate system, with inositol triphosphate, DAG, and cAMP as intracellular signals, may be involved in the mediation of ANP release (70, 132). These second messenger systems can be activated by several extracellular signals. First, calcium influx is a function of the duration of action potential, the number of action potentials, and the number of activated calcium channels. This means that ANP release may be promoted by the electrical impulses originating regularly in the sinoatrial node causing membrane depolarization and calcium influx. Cyclic AMP's role may be to increase the number of calcium channels activated by depolarization, thus enhancing calcium influx (132). The autonomic nervous system and circulating hormones such as epinephrine and vasopressin also effect these second messenger systems. These neural and hormonal factors may have their effect on ANP release directly by affecting heart rate and

contractility (99, 132). Finally, cytosolic calcium concentration depends in part on the resting length of myocardial fibers, with cytosolic calcium being increased with increased stretch (99). Tension of the atrial wall seems to regulate ANP secretion, and this may in turn be related to increased cytosolic calcium in conditions of elevated atrial stretch (99, 132).

e. ANP Receptors, Second Messengers, and Clearance

Before discussing the many actions of ANP, it seems important to mention the location and types of ANP receptors, the second messenger systems, and mechanisms of ANP clearance.

The biological actions of peptide hormones, such as ANP, are initiated by the binding of the hormone to specific glycoprotein receptors in the plasma membrane. This binding then results in the formation of an intracellular second messenger which mediates the effects of the hormone (102). Using radioligand binding and radioautography localization studies, ANP receptors have been identified in a wide variety of tissues and cells, including many cell types that are not directly involved in blood pressure and blood volume homeostasis, systems in which ANP has its major actions (102). Examples of some ANP receptor sites include bovine and rat adrenal glomerulosa, rat and dog renal glomeruli and endothelial cells, human kidney cortex, human platelets, and human brain (12, 102). In the dog kidney, it has been found that the highest ANP receptor density is in the glomeruli, followed by the collecting ducts, while there are no receptors in the proximal tubule (102). Most of these ANP binding sites have a high affinity and specificity for ANP, and the structural requirements for ANP binding to its receptors are similar to those required for biological activity (12).

There appear to be at least two major ANP receptor types, both with a molecular size of 120-140 kDa. One of these receptor types consists of a single 120-140 kDa protein, while the other is made up of two identical 60-70 kDa subunits joined by a disulfide bond (102). It is possible that additional receptor subtypes exist (20). It has also been found that the two major ANP receptor types are functionally distinct. The nonreducible 120-140 kDa receptor (ANP-R1) is linked to particulate guanylate cyclase, and when ANP binds to these receptors, cyclic guanosine monophosphate (cGMP) accumulates in the cell. The more abundant reducible 120-140 kDa receptor that is made up of two subunits (ANP-R2) is not coupled to guanylate cyclase or cGMP accumulation (102). The role of cGMP as a second messenger in mediating the actions of ANP will be discussed below.

Like most hormone-receptor systems, ANP receptors in target tissues are regulated inversely with changes in plasma ANP concentration (12). In other words, receptor down-regulation occurs when plasma ANP is increased. This phenomenon has been most extensively studied in cultured vascular smooth muscles, and in all of these studies down-regulation of ANP receptors occurred without any change in ANP receptor affinity (102). In vivo it has been found that glomerular ANP receptor density is increased in rats fed a low salt diet, which had decreased ANP levels. Conversely, rats fed a high salt diet had increased plasma ANP levels and decreased numbers of ANP receptors (12, 102).

It is also possible that the ANP receptor system may be altered by changes in the ratio of the ANP-R1 and ANP-R2 receptor subtypes. This possibility has not been directly investigated, but studies correlating the number of ANP receptors with ANP stimulation of cGMP suggest that such changes may occur, and the ratio of ANP-R1 to ANP-R2 receptors may actually be more important than total receptor number in

determining the biological response to ANP (102). Once specific agonists and antagonists for each receptor subtype are developed, a better understanding of the physiological roles for the ANP receptor subtypes will be possible (102).

In order to fulfill the criteria of a second messenger, Hamet (69) states that cGMP should be generated prior to the onset of the biological actions of ANP. This criterion has been shown to be fulfilled because low doses of ANP lead to increases of cGMP without causing significant diuresis in the subjects (69). Along similar lines, it has also been found that in conditions in which endogenous ANP levels are elevated, plasma and urine cGMP levels are also increased (69, 164).

Although a great deal has been learned about ANP synthesis, secretion, and biological actions, relatively little is known about its degradation and elimination from the circulation. The half-life rates of ANP clearance have been reported to be from 15 to 31 seconds in rats to 2.5 to 2.95 minutes in humans (35), and it has been shown that the ANP-R2 receptors, which are not coupled to guanylate cyclase, seem to serve as specific clearance binding sites for ANP (3). Hydrolysis of ANP by circulating proteases does not appear to play a major role in ANP degradation because plasma hydrolysis of ANP in vitro is quite slow (3). Almeida et al. (3) report that it is likely that the majority of ANP metabolism is due to the internalization of the ANP-R2 receptor-ANP complex, delivery of the ligand to lysosomes, and subsequent efflux of the hydrolytic products from cells to plasma (Figure 4). Since ANP-R2 receptors are widely distributed in the body and have a high affinity for ANP, they provide a very fast and effective mechanism for removing ANP from the circulation (3).

In order to determine which organs and tissues are primarily involved in ANP clearance, studies have been done to identify the tissue uptake of [^{125}I]-ANP and the clearance of

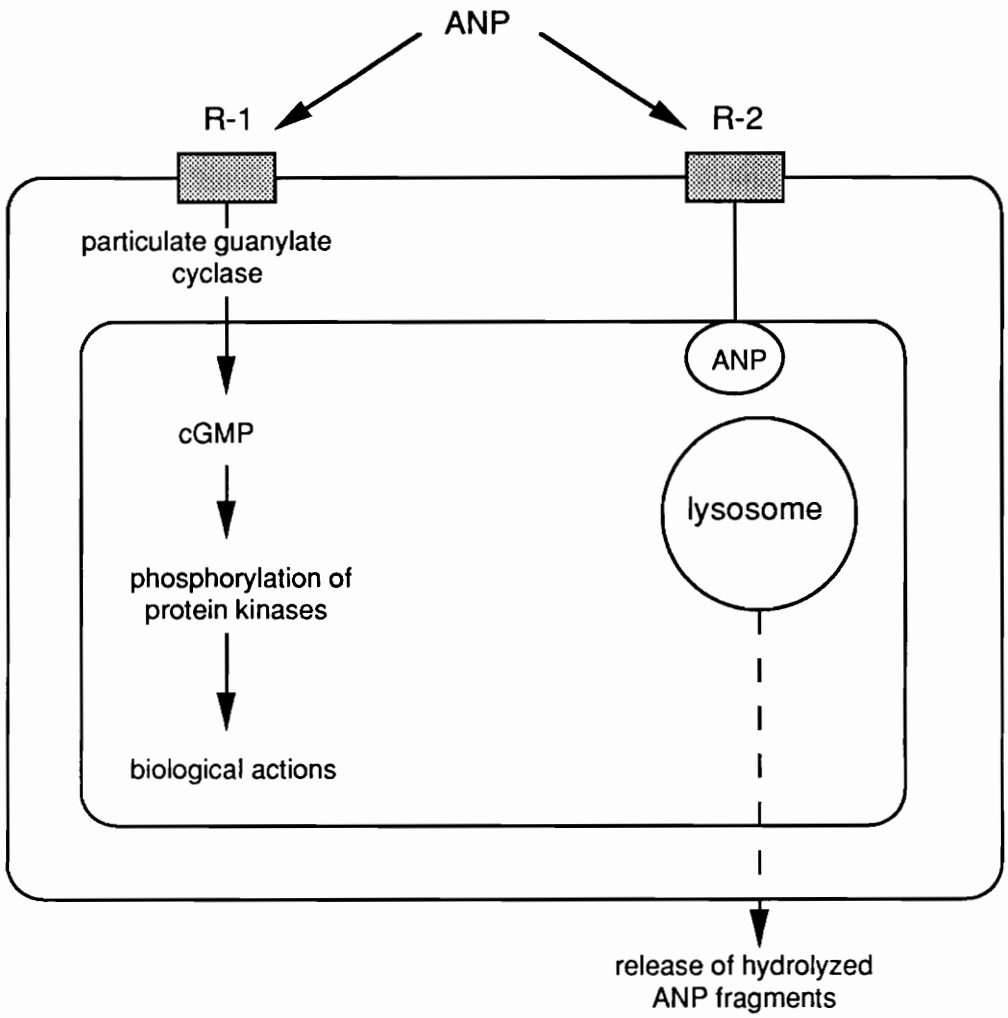

FIGURE 4: ANP receptor systems. R-1 symbolizes the ANP receptor which is coupled to particulate guanylate cyclase and is responsible for producing the biological actions of ANP. R-2 represents the ANP receptor which is involved in clearance of ANP from the blood.

ANP by certain vascular beds (79, 166). These studies have indicated that the lung, liver, and kidney are the primary organs involved in clearance of ANP (79, 166), though ANP uptake occurred in every tissue studied (166). The lungs were found to contain the highest concentration of ANP binding sites (166), and accounted for the greatest clearance (79). The release of ANP into the blood as it passes through the left heart compensates for the clearance of ANP by the lungs (79). The finding that the kidneys and liver are also major contributors to ANP clearance may help explain the increases in plasma ANP seen in renal failure and liver cirrhosis (79), although this has not been proven. More detailed study at the intracellular level will be necessary to further define the complex processes involved in ANP elimination (166).

f. ANP Actions

Atrial natriuretic peptide has actions on a wide variety of body systems to influence body fluid homeostasis and blood pressure (108) (Figure 5). These actions will be reviewed individually.

1) Renal function

The continuous administration of synthetic ANP leads to a sustained diuresis and natriuresis, along with less marked increases in calcium, magnesium, phosphate, and potassium excretion (9, 25, 108). This effect has been noted with pharmacological doses of ANP, as well as doses which maintain ANP levels in the physiological range (4).

ANP appears to have both direct and indirect effects on renal fluid handling. To identify portions of the nephron on which ANP has direct effects, the nephron segment must satisfy the following three criteria: a) have biological (ANP-R1) ANP

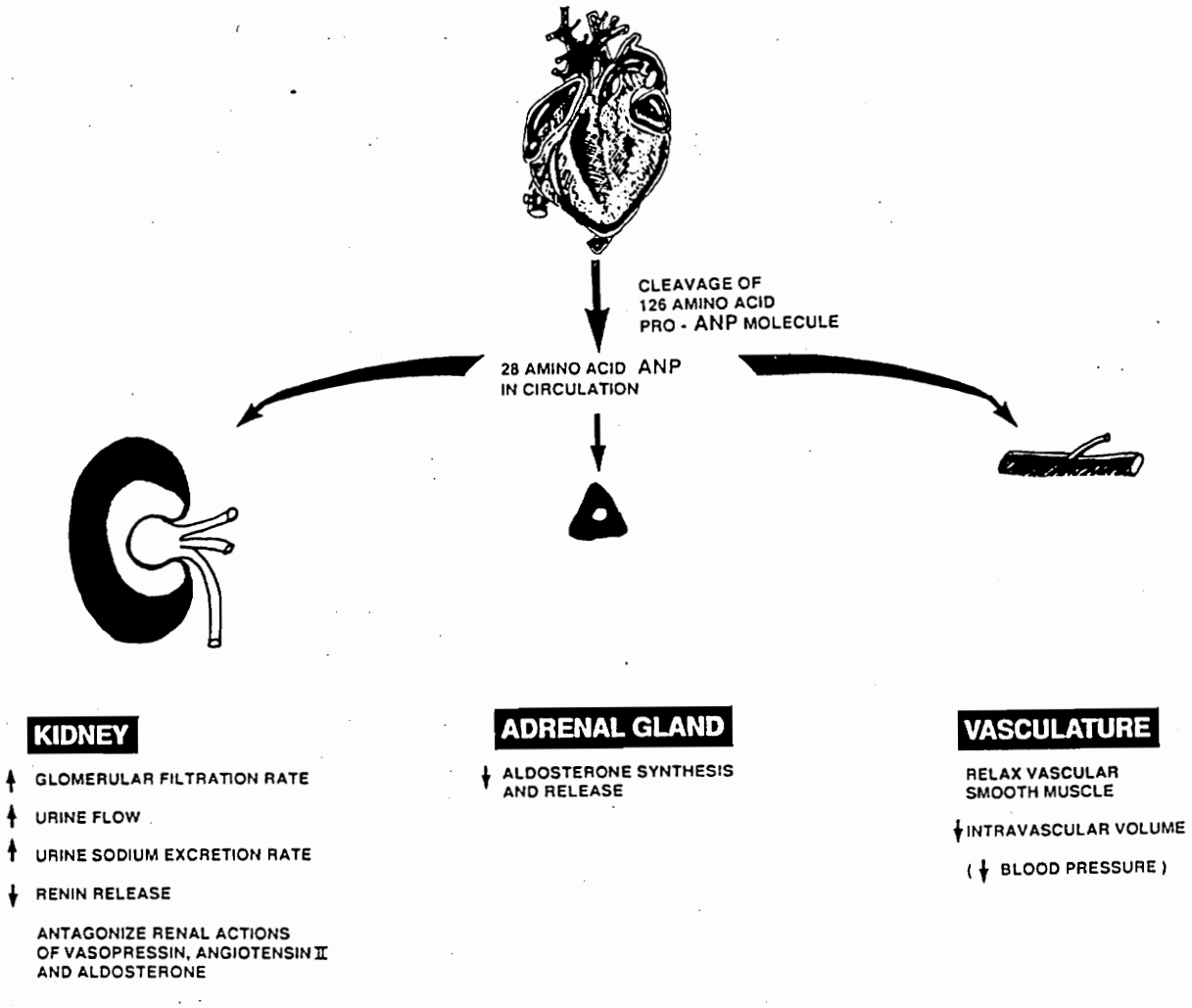

FIGURE 5: Summary of circulating ANP actions.

receptors; b) generate the second messenger cGMP in response to ANP; and c) respond functionally when exposed to ANP (32). With these three criteria in mind, ANP's proposed mechanisms of action in the kidney will be presented.

First, ANP has a marked effect on renal glomerular hemodynamics by increasing glomerular filtration rate (GFR) and filtration fraction (9, 26, 32). The renal glomerulus has the greatest ANP receptor density and generates the most cGMP in response to ANP (32), so it is not surprising that this is a major site of ANP action. ANP increases GFR by raising the glomerular capillary hydraulic pressure, and this is accomplished through dilation of afferent arterioles and constriction of efferent arterioles (32, 172). In addition, ANP increases the glomerular permeability coefficient (K_f), probably through relaxation of mesangial cells, which increases the surface area for filtration (172). It has been shown in several studies that ANP causes an increased GFR and filtration fraction without altering the absolute or regional distribution of renal blood flow (32). Other studies have shown that total renal blood flow can be reduced in animals undergoing natriuresis in response to ANP (20). Brenner et al. (20) state that this variability of response is not unexpected since changes in sympathetic nervous tone and the circulating levels of vasoconstrictors can modify vascular responsiveness to ANP. These findings indicate that changes in renal blood flow alone are not the only factors responsible for enhanced renal solute excretion in response to ANP (20).

Next, ANP has direct actions on tubular transport in the nephron. The primary site of ANP action in the tubules is in the collecting ducts, where it inhibits net sodium and water reabsorption (172). It appears to do this by altering transepithelial driving forces, acting directly on the tubular cells, and indirectly by altering the effects of aldosterone, vasopressin, and angiotensin II (32, 172). In the collecting

system, the highest density of ANP-R1 receptors and cGMP formation is in the inner medullary collecting ducts (IMCD). The proximal tubule lacks ANP-R1 receptors, has no particulate guanylate cyclase, and does not generate cGMP in response to ANP. Consistent with these findings, most studies have shown that ANP has no effects in the proximal tubule (32). However, there have been some studies that do indicate that ANP has an effect on proximal tubule ion transport (20). To explain these findings in light of the lack of ANP-R1 receptors in this region, it has been suggested that ANP acts through an intermediary pathway in the proximal tubule. Ortola et al. (116) have recently provided evidence that dopamine may mediate ANP actions in the proximal tubule. These studies showed that dopamine receptor blockade significantly blunted low-dose ANP-stimulated sodium and phosphate excretion. Cells in the loop of Henle are also devoid of ANP-R1 receptors, show only slight cGMP accumulation in response to ANP administration, and studies have shown no effect of ANP on sodium chloride transport in either the thin descending limb or the thick ascending limb (32).

ANP has a number of indirect effects on tubular transport, as well. It inhibits the synthesis of aldosterone and the release and action of vasopressin, and it antagonizes the actions of angiotensin II (32). Aldosterone stimulates sodium reabsorption in the CCD and IMCD, so over time, ANP will indirectly reduce sodium reabsorption in these segments (172). Vasopressin stimulates water reabsorption in the CCD and IMCD, so ANP can indirectly inhibit water reabsorption through its effects on vasopressin action (172). Angiotensin II can stimulate sodium reabsorption in the proximal tubule, and ANP antagonizes this action (32) (Figure 6).

There has been considerable controversy as to whether ANP exerts its effects in the kidney primarily by increasing GFR, or by its tubular actions (20, 32). There seems to be ample

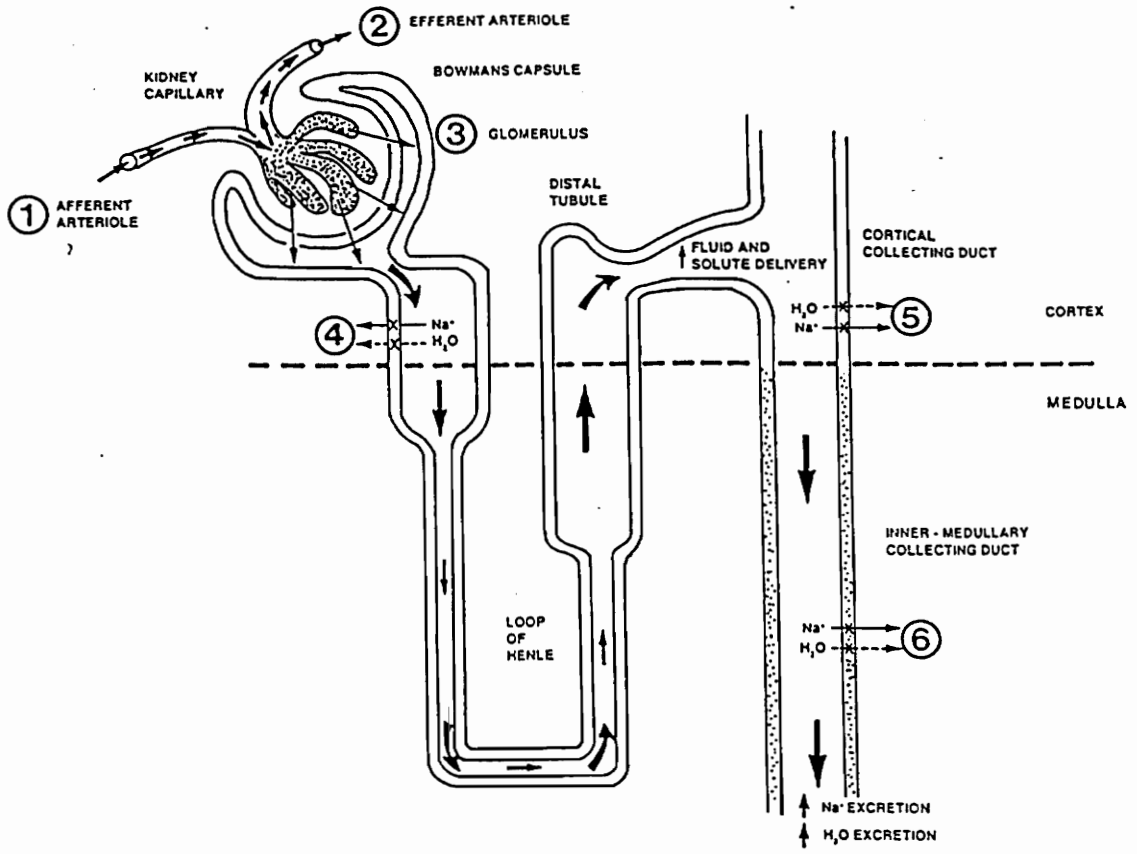

FIGURE 6: ANP actions in the kidney . Actions of ANP along the nephron include: 1) dilation of afferent arterioles; 2) constriction of efferent arterioles; 3) increased GFR and filtration fraction; 4) inhibition of sodium reabsorption in the proximal tubule through interaction with dopamine and antagonism of angiotensin II; 5) indirect inhibition of sodium and water reabsorption in the cortical collecting duct by inhibiting aldosterone and vasopressin secretion, respectively; 6) direct inhibition of sodium and water reabsorption in the inner medullary collecting duct (IMCD). Indirect inhibition by effects on aldosterone and vasopressin levels also occurs here. Dots represent ANP-R1 receptor density, which is greatest in the glomerulus and IMCD. Adapted from Zeidel (172).

evidence that ANP has actions in each of these areas which contribute to the overall natriuresis and diuresis. In summary, in the kidney ANP causes an increase in GFR and filtration fraction, and it inhibits net salt and water reabsorption in the cortical and inner medullary collecting ducts. ANP also inhibits aldosterone, angiotensin II, and vasopressin actions, thus enhancing sodium and water excretion. The result of these actions is the production of a profound diuresis and natriuresis.

2) Cardiovascular function

ANP has been shown to be an endothelium-independent vasodilator of large arteries, such as the aorta and renal artery, a dilator of certain precontracted microcirculatory vessels, and a constrictor of the postglomerular (efferent) arterioles in the kidney (154). Most veins fail to relax in response to ANP in vitro (37). ANP-R1 receptor distribution follows a similar pattern, and likewise, guanylate cyclase and cGMP have been shown to increase with the relaxation of vascular smooth muscle in response to ANP (37).

ANP relaxes blood vessels precontracted with a variety of receptor-mediated agonists, including serotonin, histamine, methoxamine, and norepinephrine, with similar potency, but it appears to have a greater effect on vessels precontracted with angiotensin II (154). Smooth muscle cell contraction requires an increase in cytosolic calcium, so it is thought that cGMP, as the second messenger for ANP, interferes with this intracellular ionic process. The studies which have been conducted in this area indicate that ANP activates smooth muscle plasma membrane Ca^{2+}-ATPase activity via stimulation of cGMP-dependent protein kinase, thus enhancing removal of calcium from the cell. ANP may also affect calcium release from intracellular stores, but this remains to be established (20).

Vascular responses to ANP infusion in vivo are less clear cut. There are numerous conflicting reports regarding the effects of ANP infusion on renal, mesenteric, coronary, femoral, and iliac blood flows and vascular resistances (12). This variability in response is likely due to species differences, dosage differences, and differing hemodynamic states (32). Thus, clear understanding of the physiologic role of endogenous ANP in regulating regional hemodynamics will not be possible until specific vascular ANP antagonists are developed (12).

Acute infusion of pharmacological doses of ANP into normo- and hypertensive animals and humans often leads to a marked reduction in mean arterial blood pressure (MABP) (154). Chronic infusions of ANP at physiological doses can also have a hypotensive action (119). The mechanisms by which ANP reduce MABP include decreased cardiac output, reductions in total peripheral resistance, and decreased intravascular volume (20). The contribution of each of these mechanisms varies with the basal condition of the experimental subject, as other factors that are involved in blood pressure regulation, such as autonomic tone, volume status, and circulating levels of angiotensin and norepinephrine, also have a major impact on overall cardiovascular response to ANP (20).

Acutely, infusions of pharmacological doses of ANP in dogs and sheep lead to a decrease in stroke volume and cardiac output (20). Heart rate has usually been shown to either decrease or remain unchanged, despite the decrease in MABP (20), though studies in humans have shown a reflex tachycardia (154). This initial ANP-induced decline in cardiac output is due in part to effects on the parasympathetic and sympathetic nervous systems. In rats, it was demonstrated that atrial extracts induce marked decreases in MABP, heart rate, stroke volume, and cardiac output by stimulating vagal afferents because prior vagotomy was associated with a lesser decrease

in MABP, no change in heart rate, and an increase in stroke volume. The fall in MABP tends to stimulate sympathetic nerve activity, but stimulation of the vagal afferents opposes this action (20).

The decrease in cardiac output which results from acute ANP infusion is also partly due to decreased preload, which is measured as decreased central venous pressure, decreased right atrial pressure (20), or decreased left ventricular end diastolic diameter (LVEDD) and decreased left ventricular end diastolic pressure (LVEDP) (121). The decrease in right atrial filling and LVEDD and LVEDP which lead to the decline in stroke volume and cardiac output must be secondary to dilation of capacitance veins, increased resistance to venous return, and/or transudation of plasma from intravascular to extravascular sites (113). Patel et al. (121) reported that the decrease in stroke volume occurs solely due to altered filling via the length-tension (Frank-Starling) relationship and is not due to negative inotropic effects of the peptide. Several studies have shown that ANP did not alter venous compliance, but did decrease central venous pressure, so the decreased central venous pressure must have been due to a decrease in intravascular volume. Accordingly, acute ANP infusion results in an increase in hematocrit and plasma protein. Diminished central venous pressure often precedes major urinary volume loss and is seen in anephric subjects, so the decreased intravascular volume must be due to redistribution of fluid from the intravascular to the extravascular space. This fluid shift could be the result of increased capillary permeability or increased transcapillary hydraulic pressure gradients, but there is conflicting evidence regarding which of these mechanisms is involved (20). It has been suggested that the distribution of ANP-R1 receptors on endothelial cells may indicate that ANP can induce a selective increase in capillary hydraulic permeability by an effect on

the endothelial lining (9). Regardless of the mechanism it appears that preload is reduced by a decrease in intravascular volume, not by dilatation of capacitance veins, in response to acute infusions of pharmacological doses of ANP (20).

Regarding the acute effects of ANP on peripheral resistance, again there are conflicting reports. It has been difficult to conclusively demonstrate ANP-induced vasodilation in the vessels that regulate peripheral resistance, namely medium-sized arteries (20). As mentioned previously, the variation in results is likely due to differences in resting vascular and autonomic tone. Parkes et al. (119) reported that chronic (5 day) infusion of low doses of ANP in sheep led to a decrease in MABP via decreased total peripheral resistance, while cardiac output returned to baseline. They concluded that acutely, the decrease in MABP leads to increased sympathetic activity through the baroreceptor reflex, but that over time this response disappears, possibly due to resetting of the baroreceptors. This decline in sympathetic activity then allows the vasodilatory action of ANP to prevail leading to decreased total peripheral resistance. However, there are conflicting reports as to whether prolonged administration of ANP lowers MABP in normotensive subjects; some studies have shown that low doses of ANP only lower MABP in hypertensive subjects (154).

In summary, most studies of the cardiovascular effects of ANP have been conducted with pharmacological doses, so there is little direct evidence of what role physiological levels of ANP play in cardiovascular regulation. Several studies have indicated that there are no changes in MABP with acute physiological and pathophysiological levels of ANP (4, 96). Anderson et al. (4) suggest that the role of endogenous ANP may be simply to modulate the action of vasoconstrictor substances. Additional long term, low dose investigations are needed to firmly establish the cardiovascular effects of ANP

and to assess its potential as an analog for developing therapeutic agents (122). The future development of ANP-specific antagonists will also greatly aid in understanding ANP's role in the body (12).

3) In the renin-angiotensin-aldosterone system

The renin-angiotensin-aldosterone system is one of the fundamental control systems in salt, water, and blood pressure homeostasis, and there is considerable evidence that ANP may act as an endogenous physiologic antagonist to this system (91).

ANP infusion at physiologic levels in dogs and humans has been shown to decrease renal renin release and plasma renin concentrations (12, 37). Multiple pathways, both direct and indirect, are probably involved in this ANP-induced inhibition of renin release (20). ANP has been shown to inhibit renin release by primary cultures of juxtaglomerular cells. ANP also increases the amount of sodium chloride available for reabsorption in the macula densa region, and this is a situation known to decrease renin release. In addition, the dilation of the glomerular afferent arteriole which results from ANP administration should elicit an inhibitory signal from renal vascular receptors to inhibit renin release (85). ANP has also been demonstrated to inhibit sympathetic stimulation of renin release (20). The effects of ANP infusion on renin release in vivo depend in part on baseline activity of the renin-angiotensin-aldosterone system (12), and ANP-induced inhibition of renin release is greater in high renin states, such as under anesthesia, and in renin-dependent hypertension (85). When pharmacologic doses of ANP are infused the hemodynamic changes induced by ANP, such as hypotension and volume contraction, could counteract the inhibitory effect on renin secretion. Thus, in some cases, there may be no change or

increased renin secretion in response to ANP infusion (85).

By decreasing renin release, ANP indirectly decreases circulating levels of angiotensin II (12). ANP antagonizes the effects of angiotensin II by causing vasodilation of vessels precontracted with angiotensin II and by inhibiting angiotensin II-stimulated sodium reabsorption in the proximal tubules of the kidney (91). In the central nervous system ANP suppresses angiotensin II-stimulated thirst. There is considerable overlap between the location of angiotensin II and ANP receptors in the brain, kidney, and adrenal cortex, which lends further support to the idea that they are endogenous antagonists (91).

In the adrenal zona glomerulosa cells ANP has been shown to decrease both basal and angiotensin II-, potassium-, and ACTH-stimulated aldosterone secretion (12). Physiological doses of ANP also decrease aldosterone synthesis and secretion in vivo (20). The ANP-induced inhibition of aldosterone synthesis is thought to take place early in steroidogenesis, at a step involving the delivery of cholesterol to the inner mitochondrial membrane and cytochrome P-450 enzyme complex. A second, less important site of inhibition is at the step where corticosterone is converted to aldosterone (20).

Despite some conflicting reports, Johnston and coworkers (91) state that there is sufficient evidence to suggest that there is a true physiologic antagonism between ANP and the renin-angiotensin-aldosterone system that allows fine-tuning of blood pressure and volume status in the body.

4) Neuroendocrine function

Many ANP-containing nerve terminals and ANP receptors are located in the brain, which suggests that ANP may have a central neuromodulator or neurotransmitter function (26). Significant ANP binding occurs in central sites that are known

to be important in hydromineral balance and cardiovascular function (136). Consistent with this, it has been found that intracerebroventricular (ICV) injection of ANP in rats inhibits dehydration- and angiotensin II-induced water intake and can decrease salt appetite in sodium-depleted animals (136), but it has no effect on these parameters in normal rats with access to water ad libitum (83). Evidence of a physiological role for endogenous brain ANP in these events is provided by studies that show that pretreatment of animals with anti-ANP serum leads to increased water intake following ICV injection of angiotensin II (136). ICV administration of ANP also antagonizes the pressor effect of centrally administered angiotensin II (83, 136). Further interaction between central angiotensin II and ANP actions is demonstrated by the finding that ICV injection of ANP inhibits the increase in plasma corticosterone which is normally seen in response to central administration of angiotensin II (83).

Additional evidence that ANP is intimately involved in blood pressure and volume homeostasis in the body comes from the finding that ANP suppresses vasopressin secretion from the posterior pituitary (91). Systemic administration of ANP inhibits vasopressin release in response to hemorrhage and prolonged dehydration (20). ICV injection of ANP has been found to produce a dose-dependent decrease in plasma vasopressin levels (83). The site of ANP's action in inhibiting vasopressin release is unclear, but it may have a direct effect on the posterior pituitary since ANP decreases vasopressin release from isolated rat neurohypophysis (20). However, Imura et al. (83) state that systemic infusion of ANP must be given in large doses to decrease vasopressin release, which suggests that the major site of ANP action is at the hypothalamic level. It has also been found that low doses of vasopressin enhance ANP secretion, so ANP may be involved in a negative feedback system for endocrine antagonism of water

homeostasis (20).

ANP binding sites and ANP-containing nerve terminals have also been found in regions of the brain that are unrelated to hydromineral balance (136). There is a significant amount of ANP immunoreactivity in hypothalamic structures, which indicates that ANP may have a role in the secretion of anterior pituitary hormones. ANP has been shown to inhibit dopamine-stimulated luteinizing hormone releasing hormone (LHRH) release from median eminence fragments in vitro. In addition, it has been shown that ICV infusion of ANP results in diminished circulating levels of prolactin, and this effect is due to stimulation of the release of the major hypothalamic prolactin-release inhibiting factor, dopamine, into hypophyseal portal blood (136). Interestingly, the ICV injection of ANP has also been found to result in decreased levels of dopamine and its metabolites in the hypothalamus (83).

5. ANP in various disease states and therapeutic potential

The role of ANP in disorders involving volume regulation, such as congestive heart failure, hypertension, liver disease, nephrotic syndrome, acute and chronic renal failure, and diabetes mellitus, has been examined by a number of investigators (20). Most of the diseases studied are generally associated with a central hypervolemia, and correspondingly, plasma ANP levels have usually been found to be elevated (108). In most of these cases the increases in ANP have been attributed to expansion of the blood volume, but it is also possible that factors such as altered clearance and interaction with circulating antagonists may be involved (26).

In congestive heart failure (CHF) increases in plasma ANP correspond with increases in atrial stretch, and significant ventricular ANP gene expression has been noted in humans and

animal models of CHF (20). Despite the increased circulating levels of ANP, patients with CHF usually exhibit volume overload, increased preload, and increased systemic vascular resistance. However, the infusion of anti-ANP antibodies in rats with CHF led to decreased renal salt and water excretion, which implies that the fluid retention would have been worse if the ANP levels had not been elevated, though the renal response was somewhat blunted (20).

In clinical forms of hypertension it has been found that plasma levels of ANP vary widely (20). ANP tends to be higher in patients with low-renin hypertension, which may be due to a larger plasma volume in these patients (26). Most studies have shown modest ANP elevations with severe hypertension and no increase in plasma ANP with mild increases in blood pressure (26). Often, administration of ANP to hypertensive subjects leads to a greater natriuretic response (26) and may result in a more marked fall in blood pressure (154) than is seen in normal subjects, depending on the type of hypertension.

Advanced hepatic cirrhosis is marked by renal sodium and water retention, and plasma ANP levels in patients with cirrhosis have been found to be either normal or elevated (20). In response to ANP infusion, urinary cGMP levels have been shown to increase in cirrhotic patients, but a relative resistance to natriuresis was noted (161). Warner et al. (161) attribute this impaired renal response to undetermined antinatriuretic factors which antagonize ANP actions.

In patients with chronic renal failure plasma ANP levels tend to be increased in a manner that is strongly correlated with the degree of volume overload (26). In rats with reduced renal mass it has been found that infusion of anti-ANP antibodies leads to a marked reduction in sodium, calcium, and phosphate excretion under conditions in which GFR and renal plasma flow are unchanged (20). This suggests that in cases

of reduced renal mass one of the major effects of ANP is the augmentation of solute excretion.

It has been suggested that ANP may have a unique role as a therapeutic agent, particularly in critical care patients undergoing parenteral therapy (108). For example, since ANP has potent glomerular actions it could prove useful as a diuretic in aggressively treating severe volume overload in cases where tubular diuretics are no longer maximally effective. In addition, ANP has been shown to antagonize norepinephrine and gentamicin-induced acute renal failure in the rat, so ANP may prove beneficial in treating this condition clinically (37). In order for ANP to become routinely used clinically analogs with a longer duration of action and the capability of nonparenteral administration will need to be developed (108). Intranasal administration of ANP has been attempted, but there was no evidence of absorption and action (24). It is not clear whether ANP will ever be used therapeutically, but the idea is intriguing, and it would be premature to rule out the possibility (24).

2. Diabetes Mellitus

Diabetes mellitus (DM) is a complex metabolic disorder. In its fully developed form in the human it involves fasting hyperglycemia, micro- and macroangiopathic vascular disease, retinopathy, nephropathy, and neuropathy (127). It is characterized by an absolute or relative insulin deficiency, the degree of which determines the subtypes of Type 1 (insulin-dependent) and Type 2 (noninsulin-dependent) DM (127). This review will focus on Type 1 DM in humans and animal models of DM because a model of Type 1 DM was studied in this investigation.

a. Etiology and Pathogenesis of Type 1 DM

The etiology of Type 1 DM in man is complex and incompletely understood. Type 1 DM is characterized by severe insulinopenia which results from the destruction of the majority of insulin-secreting beta cells in the islets of Langerhans in the pancreas (127). During the last few decades genetic factors, autoimmunity, and viral infections have been extensively studied as possible causes of beta cell destruction (169). In part as a result of these studies, the pathogenesis of Type 1 DM has been divided into six stages: 1) Certain individuals are genetically predisposed to developing the disease; 2) a triggering event then leads to active autoimmunity of beta cells; 3) the autoimmune response causes a decline in the beta cell mass; 4) over years, there is progressive loss of beta cells leading to diminished insulin secretion in response to an intravenous glucose tolerance test, though the patient is still normoglycemic; 5) overt DM results when approximately 90% of the beta cells are lost. Some residual insulin production can still be demonstrated by the presence of the connecting peptide of proinsulin (C peptide) in the serum, but the patient requires exogenous insulin therapy; 6) C peptide is no longer found in the circulation, and all beta cells are assumed to be destroyed (173).

1) Genetic factors

It has been suspected for some time that there is a heritable component to the development of Type 1 DM because the disease has been noted to cluster in families (173). Genetic influences have been found to be important, but not crucial to the development of the disease, because only approximately 36% of identical twins of diabetic patients

develop DM (103). This low concordance rate in identical twins suggests that susceptibility to Type 1 DM is inherited rather than the expressed disease (98).

Inherited susceptibility to Type 1 DM in humans has been mapped to the major histocompatibility complex (MHC) on the short arm of chromosome six (90). The MHC comprises the chromosomal region that controls the synthesis of transplantation antigens, so it plays an important role in immune processes (127). MHC genes are classified into human leukocyte antigen (HLA) class I, II, and III loci (138), and genes in the class II region are associated with Type 1 DM in humans (103). The class II genes encode glycoproteins which occur on the surface of certain cell types, including macrophages, B lymphocytes, and activated T lymphocytes. The class II region is subdivided into the DR, DQ, and DP series (90). HLA DR3 and HLA DR4 alleles and HLA DQ genes linked to these DR genes appear to be associated with Type 1 DM, particularly in Caucasians and American black populations (103). It has also been found that the HLA DR2 allele seems to be protective against the development of Type 1 DM in many cases (103). In addition to the HLA-DR and DQ genes, it is possible that non-MHC genes may be important in determining the inherited susceptibility to the disease, but little is known of their location (90). Progress in defining the genetics of Type 1 DM will lead to a better understanding of the pathogenesis of the disease (90).

2) Autoimmune factors

Type 1 diabetes in humans is considered by many to be a genetically programmed autoimmune disease (52). The clinical onset of Type 1 DM is characterized by both cellular and humoral immune changes (103). The presence of islet cell antibodies (ICA) in the serum is strongly associated with the development of Type 1 DM and may precede the development of

overt diabetes by up to nine years (52). In addition, insulin antibodies appear in approximately 30% of those tested who later develop Type 1 DM, and 80% of those with insulin antibodies also have islet cell antibodies (52). However, it is unlikely that these changes in humoral immunity are the major determinants of beta cell destruction. Most ICA's are not specific for beta cells, and DM cannot be induced in animal models by inoculation with these antibodies (52). Eisenbarth (52) states that there is evidence that T lymphocytes are the major determinants of beta cell destruction. It has been found that T lymphocytes from patients with Type 1 DM can inhibit insulin secretion by rat beta cells. These activated T cells acquire a series of cell surface antigens, including antigens encoded by HLA class II molecules. However, there is insufficient evidence to assert that activated T lymphocytes cause diabetes, since their specificity for beta cells has not been established (103).

Recently, two additional targets for autoantibody-mediated destruction of beta cells have been reported. Maclaren et al. (106) state that the discovery of a 64 kDa unique beta cell membrane protein probably represents the best candidate for a primary autoantigen. This protein is nearly universally present in prediabetic individuals, although its functions are obscure (106). The second newly discovered potential mediator of beta cell destruction is an autoantibody that reacts with polar antigens in radiation-induced insulinoma (RIN) cells (173). This antibody is found in approximately 30% of humans with recent onset Type 1 DM and prediabetic individuals (106). The function and predictive value of these anti-polar antibodies is currently under investigation (173).

There appears to be ample evidence that abnormalities in immune function are involved in the destruction of the beta cells in Type 1 DM in humans, but much remains to be learned in this area.

3) Viral and other environmental factors

In at least some cases, Type 1 DM seems to be the result of environmental agents acting in genetically susceptible individuals (103). Viruses, toxic agents, and dietary triggers of autoimmunity are the environmental factors which have been considered, with viruses receiving most of the attention (103, 173). Of the possible toxic triggers of beta cell destruction only N-nitroso derivatives such as alloxan, streptozotocin, and the rat poison Vacor are known to cause DM (103).

The evidence for a viral etiology in Type 1 DM comes primarily from animal studies, but there is some evidence from human studies as well (169). Yoon (169) states that there are two possible mechanisms for virus-induced DM. The virus can have a direct cytotoxic effect on the beta cells, or beta cell autoimmunity may be triggered by certain viruses. In animals, the encephalomyocarditis virus, Mengovirus-2T, and Coxsackie B virus can directly infect murine beta cells, where they replicate and destroy the cells (169). In man there is no direct evidence that cytolytic viral infections can induce DM, although Coxsackie B viruses can infect human beta cells in vitro, and this virus has been isolated from the pancreatic tissue of some children dying of acute onset Type 1 DM (169). The rubella virus and congenital rubella syndrome (CRS) provide the most evidence that a virus can trigger autoimmune-mediated beta cell destruction in man. In people with CRS, DM can develop 5-20 years after birth, it is linked to specific HLA haplotypes, and it may be associated with the production of autoantibodies to islet cells and thyroid tissue. Case studies of children with CRS show that approximately 20% develop Type 1 DM, but it is not known how the rubella virus triggers autoimmunity and DM (169). The mumps virus and cytomegalovirus are two other viruses that may be associated

with triggering beta cell autoimmunity in some cases in man (169). With further study the contributions and interaction between genetic and environmental factors in the development of Type 1 DM should become more clear.

b. Symptoms and Diagnosis

Type 1 DM usually has an acute or subacute onset in youth, but it can occur at any age. There is an abrupt, severe insulin deficiency, which results in weight loss despite hyperphagia, polyuria, nocturia, polydipsia, dehydration, glucosuria, and potentially ketoacidosis and coma (127). Glucosuria results when the concentration of glucose being filtered by the glomerulus exceeds the capacity for reabsorption by the renal tubules. This occurs when the plasma glucose concentration is greater than 180mg/dL in man. Weight loss is due to the urinary loss of glucose, dissolution of adipose tissue secondary to decreased insulin levels, and uncompensated fluid loss (127). Some patients experience a remission of the disease for up to several months after initial therapy restores metabolic control. This "honeymoon" period is almost invariably transient (127).

The diagnosis of Type 1 DM is based on: 1) random plasma glucose concentration of greater than 200mg/dL along with the classic symptoms of polyuria, polydipsia, weight loss, and glucosuria or ketonuria; 2) increased plasma glucose (greater than 140mg/dL) on more than one occasion following a 10-16 hour fast; or 3) elevation in plasma glucose after an oral glucose challenge on more than one occasion. An abnormal glucose tolerance test is generally defined as a venous plasma glucose concentration greater than 200mg/dL two hours after a 75g glucose load (127). In making the diagnosis other factors which can result in fasting hyperglycemia and impaired glucose tolerance must be ruled out, such as trauma, kidney or liver

disease, or the use of certain drugs that can impair glucose metabolism (54).

c. Pathophysiology of Diabetes Mellitus

1) Effects of insulin and lack of insulin on carbohydrate, protein, and lipid metabolism

Insulin is a polypeptide hormone consisting of 51 amino acids in two chains that is produced by the pancreatic beta cells (127), which make up approximately 60% of the cells in the islets of Langerhans (6). It is the primary factor controlling the storage and metabolism of ingested nutrients, and insulin action involves the three major fuels of carbohydrate, protein, and fat with actions in three principle tissues, namely, liver, skeletal muscle, and adipose tissue (143). The metabolic alterations noted in DM are a reflection of the degree of insulin deficiency. Acute, severe insulin deficiency leads to underutilization of glucose by skeletal muscle, adipose tissue, and the liver and overproduction of glucose by the liver via gluconeogenesis and glycogenolysis (8). Insulin enhances the transport of glucose across cell membranes in skeletal muscle and adipose tissue, so lack of insulin leads to underutilization of glucose in these tissues. In the liver hepatic cells do not retain glucose in the face of insulinopenia because glucokinase, the enzyme necessary for the first step in glucose metabolism, is deficient with decreased insulin (8). The first step in glucose metabolism is phosphorylation of the molecule, and without phosphorylation the glucose molecule that freely enters the hepatocyte flows out again. In addition, with diminished levels of circulating insulin glycogenolysis is initiated in the liver by activation of a series of enzymatic processes that remove glucose molecules from glycogen polymers. This increases the

hyperglycemia that already exists due to the decreased glucose uptake by the peripheral tissues (8).

Insulin has major effects on protein metabolism, as well as carbohydrate metabolism. In the healthy individual the branched chain amino acids (leucine, isoleucine, and valine) preferentially escape hepatic uptake and metabolism after absorption following a protein meal, and they account for much of the amino acid uptake by muscle (143). With insulin deficiency, however, amino acid uptake by skeletal muscle is decreased and proteolysis occurs (8). The plasma levels of branched chain amino acids are increased, and the glucogenic amino acids alanine and glycine are released from muscle, transported to the liver, and used for gluconeogenesis, thus again adding to the existing hyperglycemia (8). Insulinopenia also results in decreased protein synthesis (48, 167). The decreased protein synthesis is thought to be due to a slowed rate of mRNA translation secondary to deficiencies in peptide chain initiation and elongation and a decreased number of ribosomes available for mRNA translation (48). In cardiac muscle diminished protein synthesis is partially due to impaired mRNA translational efficiency, as well as to the other factors noted in skeletal muscle (48, 167). Proteolysis is increased with insulin deficiency, and this can lead to large increases in urinary nitrogen excretion and a negative nitrogen balance. Muscle mass is lost, and there is muscular weakness (8).

The third major area of metabolic derangement in Type 1 DM involves lipid metabolism. With insulinopenia lipolysis is accelerated and lipogenesis is decreased (50). As insulin levels decrease the adenyl cyclase system in the membrane of adipocytes is activated, and with the resulting formation of the second messenger cyclic AMP, intracellular hormone-sensitive lipases are activated. These enzymes then facilitate the hydrolysis of triglycerides within the adipocytes to

free fatty acids (FFA) and glycerol. The concentration of FFA's in the plasma increases, and the liver oxidizes the excess FFA's to acetoacetyl CoA and acetyl CoA, which are the building blocks for the ketone bodies B-hydroxybutyric acid and acetoacetate (8). These ketone bodies are not readily metabolizable without insulin, so they accumulate in the plasma, and when this occurs to excess it results in metabolic acidosis. Some of the FFA's presented to the liver are re-esterified to triglycerides and packaged as very low density lipoproteins (VLDL), which diffuse into the circulation (8). With insulin deficiency lipoprotein lipase activity is diminished, and this enzyme is needed for removal of VLDL from the plasma and for metabolism of the lipoprotein, chylomicron (8, 50). Thus, hyperchylomicronemia may also be noted in DM. In addition, diabetics whose glucose levels are poorly regulated may have elevated plasma levels of total cholesterol and low density lipoprotein (50).

Alpha cells are the second most abundant cell type in pancreatic islets, and these cells produce the hormone glucagon. Abnormalities in alpha cell function are noted in Type 1 DM, along with the major beta cell dysfunction (143). Liver is the primary target tissue for glucagon, where it stimulates glycogenolysis, hepatic amino acid trapping, and ureagenesis (6). Normally, glucose inhibits glucagon secretion, but this suppression is lost in DM, and protein-stimulated glucagon secretion is augmented (143). Increased glucagon levels seem to contribute to the diabetic state primarily in times of insulin deficiency, but have no effect on glucose tolerance as long as insulin is available. When insulin is deficient the elevated glucagon levels add to the hyperglycemia and can accelerate the development of keto-acidosis (143).

Even with rigorous insulin therapy it is difficult to restore perfect metabolic control in patients with Type 1 DM.

The consequences of long term aberrations in carbohydrate, lipid, and protein metabolism are profound, and they contribute to the myriad of long term diabetic complications (8).

2) Renal complications

Diabetic nephropathy is one of the most common disabling complications of diabetes mellitus (158), and in the United States it accounts for 25% of the patients on chronic renal dialysis (112). Thirty to 40% of people who develop Type 1 DM before the age of 35 later develop diabetic nephropathy, which is identified by persistent proteinuria, hypertension, and progressive loss of renal function (113).

Diabetic nephropathy is characterized by a number of renal structural and functional changes as the disease progresses. Typically, the early stages of Type 1 DM in humans and experimental DM in animals are characterized by glomerular hyperfiltration (5). In newly diagnosed juveniles with Type 1 DM the GFR is increased by an average of 40% (113). Renal plasma flow may also be increased (158). The increased GFR may persist for years in some patients, but in those who develop nephropathy the GFR begins to decline approximately 10-20 years after the onset of the signs of DM (113). Long before the development of overt diabetic nephropathy with persistent proteinuria, small increases in albumin excretion, called microalbuminuria, occur in people with DM (112). This decline in GFR is accompanied by clinically evident persistent proteinuria (158). Early structural changes include enlargement of the kidneys with hypertrophy of individual glomeruli (158) and an increase in glomerular capillary surface area (113). Within a few years a widening of the glomerular basement membrane (GBM) can be detected, followed by an increase in the fractional volume of both the cellular and matrix components of the mesangium (146).

Enlargement of the mesangium occurs at the expense of glomerular capillary luminal space and filtration surface area (146). Patients with established diabetic renal disease exhibit diffuse or nodular glomerulosclerosis, leading to progressive capillary occlusion, with the diffuse form being most common (158). Renal arteriolar hyalinization may also occur at this stage, and it is considered a hallmark of diabetic nephropathy (113).

In rats with experimentally-induced DM by streptozotocin (STZ), alloxan, or pancreatectomy, it has been shown that kidney weight increases approximately 20% within three days of diabetes induction (71). If the rats do not receive insulin therapy, within 6-9 months GBM thickening and increased mesangial volume are noted. Diffuse glomerulosclerosis has been found in these rats after 9-27 months of DM, but not to a degree sufficient to cause significant capillary occlusion. The nodular form of glomerulosclerosis has not been observed in experimentally-induced diabetes in rats (71). In addition, no significant renal arteriolar hyalinization has been reported in moderately hyperglycemic diabetic rats (113). There is little evidence of the progressive decline in renal function that is so common in human Type 1 DM (71). Significant proteinuria is not found in these models (71), nor is it found in the spontaneously insulin-dependent diabetic BB rat (158). Thus, while the early functional and morphological renal alterations in diabetic humans and rats are very similar, it appears that there are significant differences between the two in the late stages of DM, and the usefulness of the rat models in studying final phases of diabetic nephropathy should be questioned (71).

The pathophysiological mechanisms of the glomerular hyperfiltration seen in the early stages of Type 1 DM have not been fully elucidated, but a large number of theories abound. Among the possible mediators of hyperfiltration in DM are

increased renal plasma flow and increased glomerular capillary pressure (159), hyperglycemia, increased plasma levels of glucoregulatory hormones (glucagon, growth hormone), augmented glomerular production of vasodilator prostaglandins, increased plasma levels of ANP (158), increased protein intake, renal hypertrophy (113), and increased activity of the polyol pathway and disturbances in myo-inositol metabolism (5). The glomerular hyperfiltration can be decreased through optimal glycemic control (5), dietary protein restriction (113), administration of cyclooxygenase inhibitors (112), infusion of anti-ANP antibodies (117), and inhibition of the polyol pathway (158). These findings suggest that each of the proposed mediators of glomerular hyperfiltration probably contributes to the elevated GFR found in the early stages of DM, with no single factor being the sole cause.

The pathogenesis of the renal lesions in diabetic nephropathy which ultimately lead to end stage renal failure are not fully understood. A number of factors are probably involved in the formation of the renal lesions and hyperglycemia, hemodynamic alterations, and hypertension are the factors which have received the most attention.

The notion that hyperglycemia may be involved in the development of renal GBM thickening and mesangial expansion stems in part from the fact that increased blood glucose leads to an increase in the nonenzymatic glycosylation of proteins. Increased glycosylation of the GBM has been found in rats with experimentally induced DM (158). This may precipitate changes in the composition and synthesis of GBM leading to structural and functional renal alterations.

Hemodynamic changes appear to be critically important in producing glomerulopathy. Increased glomerular capillary pressure (Pgc) is postulated to increase the filtration of plasma proteins and other macromolecules into the urinary space and mesangium and to secondarily stimulate the produc-

tion of mesangial and basement membrane components (158). The importance of increased Pgc in the development of diabetic nephropathy is suggested by investigations in diabetic rats which have demonstrated that both dietary protein restriction and angiotensin converting enzyme (ACE) inhibitors, such as enalapril and captopril, prevent glomerular hypertension as well as proteinuria and progressive loss of renal function (112). Several recent studies in human diabetic patients with nephropathy have also shown that captopril (113) and dietary protein restriction (5) seem to slow the progressive decline in renal function. The mechanism by which ACE inhibitors work to decrease Pgc is uncertain, but it may lie in the ability of these compounds to lower efferent arteriolar resistance (112).

Hypertension in DM contributes to the acceleration of complications in man, and it has been shown to develop directly as a consequence of DM, not secondarily to diabetic nephropathy, as commonly thought (5). This is demonstrated by the fact that increased systemic blood pressure often precedes the onset of proteinuria and renal insufficiency by many years (112). Systemic pressure is a determinant of glomerular pressure (112), so it is not surprising that hypertension accelerates the rate at which renal complications worsen (5). Interestingly, experimental diabetes in the rat is not accompanied by overt systemic hypertension (5).

In summary, it is likely that a number of different factors interact to produce the renal structural and functional changes common to diabetic nephropathy. Strict metabolic control can prevent glomerular hyperfiltration in diabetic rats and man, but whether strict control from the onset of DM in man can retard the development of nephropathy is still undetermined (113). Considering the difficulties of achieving long term strict metabolic control in all DM patients, further investigation into the mechanisms of hemodynamic injury should be carried out. This may reveal nutritional and/or pharmaco-

logical interventions that will provide a good and readily achievable means for conferring protection against the debilitating complications of this disease (5).

3) Cardiovascular complications

Several cardiovascular disorders are among the long term complications associated with Type 1 DM. These complications include microangiopathy, macroangiopathy, cardiomyopathy, and hypertension.

i) Microangiopathy

Disturbance of the microcirculation is a major cause of disability and mortality in DM (77). Initially, a triopathy of retinal damage, nephropathy, and neuropathy was recognized in DM, and the term microangiopathy evolved with the observation that many other microvascular beds are also influenced by DM, including those of the heart, skin, subcutaneous fat, and muscles (152). The function of the microcirculation, which can be defined as the microscopically small blood vessels including and distal to the arterioles (152), is to transport and exchange nutrients and waste products between the blood and tissue fluid (77). The ease of passage of the exchanged molecules through the capillaries depends on the charge and size of the molecule and the size and surface charge of the interendothelial gap (152). This exchange function eventually fails in those organs clinically affected by microangiopathy (152).

The structural hallmark of microvascular damage is thickening of the capillary basement membrane (168). In normal basement membrane, Type IV collagen is the most abundant protein, and other proteins which comprise the basement membrane include the highly negatively charged

heparan sulfate proteoglycan (152) and the glycoproteins laminin, fibronectin, and entactin (168). Animal models have indicated that Type IV collagen and laminin production are increased and heparan sulfate proteoglycan production is decreased in the thickened capillary basement membranes found in DM (152). It has been shown that there is increased non-enzymatic glycosylation of the Type IV collagen in the basement membranes of the glomeruli in diabetic humans and animals (168), and it is thought that glycosylated collagen may be more resistant to normal degradation, thus allowing its build up (152). The impaired synthesis of negatively charged heparan sulfate proteoglycan in diabetic animals suggests that there is a reduced charge barrier. This would explain the apparent paradox of a thickened capillary wall which offers a reduced barrier to macromolecules, as exemplified by the microalbuminuria that accompanies diabetic nephropathy (152).

The factors contributing to the pathogenesis of these microvascular alterations is the focus of much interest. The major factors thought to be involved are hyperglycemia and its effects on sorbitol metabolism and nonenzymatic glycosylation and hemodynamic changes. The frequency and severity of capillary basement membrane changes is associated with the severity and duration of hyperglycemia (168). The data suggests that once microangiopathy is clinically evident, especially in retinopathy, it responds poorly to improved glycemic control, but there is evidence that good control early in diabetes protects against the development of this complication (152). It has also been hypothesized that early in DM increases in microvascular flow and pressure occur leading to microvascular sclerosis, which ultimately limits perfusion and causes loss of autoregulatory capacity (152). Blood flow is increased in the kidney, retina, and periphery early in DM, and blood flow is low and/or has limited ability to increase in response to maintained stimulation as the

disease progresses (152). It is thought by some that increased vascular pressure rather than increased flow may mediate hemodynamic-related injury because studies have shown that thickening of capillary basement membranes in the distal lower extremities of nondiabetic humans is strongly correlated with increased vascular pressure, while blood flow is decreased in this region. Increased tension on the vascular wall could be a stimulus for vascular cells to synthesize basement membrane (168). If hemodynamic alterations do play a key role in the pathogenesis of microangiopathy, it is important that the mechanism of initial hyperemia noted in many organs early in DM be elucidated so that therapeutic interventions can be developed (152).

Thus, it seems that hyperglycemia and hemodynamic alterations are key elements in the pathogenesis of capillary basement membrane thickening (152). The early changes seen in the microvasculature of patients with DM may be reversible, but over time secondary mechanisms may become involved which render the organ damage irreversible. At this time the most important preventative measure for reducing the incidence of microangiopathy appears to be the institution of optimal glycemic control as early as possible after diagnosis of DM (152).

ii) Macroangiopathy

Macrovascular disease in DM refers primarily to atherosclerosis. In the nondiabetic population the development of atherosclerosis is thought to progress in the following manner. The arterial endothelium is injured, perhaps by turbulent blood flow (88), and this is followed by the attachment of monocytes or macrophages to the site of injury. Subendothelial migration of the monocyte/macrophages follows, and platelets adhere to the site of injury. Platelet aggrega-

tion with the release of potent vasoconstrictors and pro-aggregatory arachidonic acid metabolites then occurs. Platelets, macrophages, and endothelial cells release growth factors that stimulate smooth muscle cell proliferation at the site. Low density lipoprotein (LDL), intermediate density lipoprotein, and very low density lipoprotein (VLDL) may deliver cholesterol to the site, while high density lipoprotein (HDL) may protect against cholesterol deposition. Altered arterial wall fibrinolysis, genetics, and humoral factors may also be involved in the process (34). In DM, the development of atherosclerosis is thought to follow a similar path, but a number of alterations observed in DM allow for atherosclerosis to develop at an accelerated rate. In developed countries atherosclerosis is the most common long term complication of chronic DM, and it culminates in such fatal complications as myocardial infarct, stroke, and gangrene (88). The most significant macrovascular complication is coronary heart disease (88), and in countries where atherosclerosis is common in the general population coronary atherosclerosis is often more extensive and severe in diabetics than in matched controls (89). The factors which contribute to the accelerated development of atherosclerosis in DM include alterations in endothelial cell function, lipid and lipoprotein metabolism, platelet function, and such contributing factors as increased vascular wall rigidity (88) and diabetic nephropathy (34). Diabetic rodent models, however, are relatively resistant to the development of atherosclerosis (29). Atherosclerosis is a very complicated phenomenon, and factors many are probably involved. Fortunately, though, careful metabolic management in patients with DM can bring many of these changes close to normal and reduce the risk of premature macrovascular disease (34). There is no evidence that ANP contributes to atherogenesis, so the mechanisms leading to this complication will not be discussed.

iii) Cardiomyopathy

There now exists a substantial body of clinical, epidemiologic, and pathologic data to support the existence of a specific diabetic form of cardiomyopathy. The frequent association of DM with coronary atherosclerosis and hypertension make it difficult to separate these potential causes of heart disease from those due to metabolic derangements alone, but it seems clear now that such a cardiomyopathy does exist (171). Data from the Framingham Heart Study indicated that diabetic men had a 2.4 fold greater risk for developing heart failure than nondiabetic men over an 18 year period, and the risk for diabetic women was 5.1 times greater (61). This increased risk persisted after accounting for age, hypertension, obesity, hypercholesterolemia, and coronary atherosclerosis. Further studies have supported these findings (171).

Prior to the development of overt congestive heart failure, a number of preclinical abnormalities can be detected in many Type 1 and Type 2 diabetic patients. Subclinical left ventricular dysfunction has been detected in diabetics using a variety of noninvasive techniques (171). These studies have found significant abnormalities in systolic function, such as decreased ejection fraction and percent fractional shortening with resultant increases in end systolic volumes in DM. The response of the left ventricle to exercise has been used extensively in diabetic subjects to detect latent cardiac dysfunction. Such studies have found that a significantly greater percentage of diabetics have an abnormal ejection fraction in response to exercise compared to controls, even with normal ejection fractions at rest. These abnormalities were not found to be caused by reduced myocardial perfusion and were not correlated to duration of diabetes or microvascular complications (171). Preclinical abnormalities in left ventricular diastolic function have also been

detected in young Type 1 diabetic subjects. Diastolic filling abnormalities, namely prolongation of early diastolic rapid filling, were identified in almost 30% of diabetic subjects by measuring transmitral flow velocities with Doppler echocardiography. The subjects in the control and diabetic groups showed no evidence of ischemic, valvular, or hypertensive heart disease, and there was no correlation of diabetic abnormalities with duration of diabetes or microvascular complications (171). Other investigations have shown significantly elevated left ventricular end diastolic pressure with diminished left ventricular end diastolic volume in diabetic patients, which is consistent with decreased ventricular compliance (90). In rats with experimentally-induced DM, diabetes results in a decreased response of cardiac output to increased filling pressure and extreme sensitivity to changes in afterload, with diminished systolic response to high levels of afterload (171).

Early clinically apparent diabetic cardiomyopathy results in increased pulmonary and systemic venous pressure, audible fourth heart sound, increased left ventricular wall thickness with decreased left ventricular wall:cavity ratio, and normal heart size (61). Decreased stroke volume and increased end diastolic pressure may occur, the electrocardiogram may show nonspecific ST-segment and T-wave changes, conduction abnormalities, and left ventricular hypertrophy. The disease may progress to resemble dilated cardiomyopathy (61).

A number of gross and histologic changes are found in diabetic cardiomyopathic hearts. Grossly, heart weight is increased, the heart is pale in appearance, and it is firm on palpation (61). In both diabetic dogs and man there is an accumulation of PAS-positive material or glycoprotein in the myocardial interstitium, and this is thought to contribute to the decreased ventricular compliance (171). Increased myocardial collagen content (61), hypertrophy, and fibrosis

are noted (171). In both rats and man the combination of DM and hypertension leads to greater interstitial fibrosis and myocellular damage than either disorder alone (171). In addition, capillary basement membranes may be thickened, and capillary microaneurysms are found, though cardiomyopathy can occur in diabetics with no evidence of vascular compromise (61). Interestingly, animal studies have shown increased triglyceride and cholesterol levels in the left ventricle despite normal serum concentrations of these compounds. This suggests that there is an abnormality in myocardial intracellular cholesterol metabolism (171). Few myocardial parenchymal alterations have been seen in normotensive rats with experimentally-induced DM, but several biochemical alterations have been detected which may account for the observed changes in systolic function and ventricular relaxation. These changes include decreased myocardial ATP-ase activity and a predominance of isoenzymes with lower ATP-ase activity. Calcium binding and uptake by the sarcoplasmic reticulum is also decreased (171).

A number of factors may contribute to the pathogenesis and pathophysiology of diabetic cardiomyopathy. Some of the elements that are thought to be involved in the development of this disorder are metabolic derangements, autonomic neuropathy, and hypertension.

As with the other complications of DM discussed, hyperglycemia probably plays a critical role in the development of diabetic cardiomyopathy as well. Hyperglycemia leads to nonenzymatic glycosylation of proteins, and it is thought that glucose-derived cross-linking of collagen may be one cause of the diminished left ventricular compliance noted in diabetic cardiomyopathy (61). Hyperlipidemia may alter cell membranes and enzyme activity by changing the phospholipid:cholesterol ratio, and this may contribute to myocardial membrane abnormalities, leading to cardiomyopathy (61).

Cardiac autonomic neuropathy is a disorder that occurs in 20-40% of diabetic patients, and it is part of a much wider spectrum of autonomic neuropathies which affect most organs (55). Since the autonomic nervous system may be involved in the release of ANP (132), it seems possible that cardiac autonomic neuropathy may affect ANP release in DM. Damage to the autonomic nerves is due in part to abnormal polyol metabolism, and both parasympathetic and sympathetic nerves are affected (61). Damage to vagal efferents leads to abnormal heart rate control, and disturbances in blood pressure regulation occur as a result of sympathetic vasoconstrictor damage (55). This leads to a failure of the baroreceptor reflex to maintain blood pressure on standing, or orthostatic hypotension (93). How cardiac autonomic neuropathy may interact with diabetic cardiomyopathy is uncertain, but several theories have been proposed. First, there is evidence that alterations in sympathetic nerve activity would alter diastolic filling (93). Catecholamines facilitate calcium uptake by the sarcoplasmic reticulum, which exerts a relaxing effect on the myocardium, thus allowing greater diastolic filling. It has been found that myocardial catecholamine depletion occurs in patients with congestive heart failure, and it has been reported that plasma norepinephrine levels are decreased in Type 1 diabetic patients with cardiac autonomic neuropathy and abnormal left ventricular diastolic filling. In normal individuals sympathetic nervous stimulation also improves left ventricular contractility. Thus, sympathetic nervous system dysfunction may affect left ventricular systolic and diastolic function (171). The increased heart rate that occurs in patients with cardiac autonomic neuropathy as a result of diminished efferent vagal input may also add to inadequate diastolic filling (61). Clearly, cardiac autonomic nervous dysfunction, in combination with the other diabetes-induced myocardial abnormalities, may

contribute to the pathogenesis of diabetic cardiomyopathy.

Finally, it should be noted that the structural and metabolic changes which occur in the heart as a result of systemic hypertension closely resemble those found in diabetic cardiomyopathy. When hypertension and DM coexist, a more severe degree of cardiomyopathy can result, though the mechanisms of interaction are unclear (61). This provides yet another reason why hypertension should be strictly controlled in diabetic patients.

In summary, there is a substantial body of evidence to support the existence of a specific diabetic cardiomyopathy. The pathogenic significance of the many factors that may affect myocardial performance in the diabetic awaits further clarification (171).

iv) Hypertension

Hypertension, which is generally defined as systolic pressure of greater than 160mmHg and/or diastolic pressure of greater than 95mmHg in humans, is approximately 1.5-2.0 times more prevalent in persons with diabetes as compared to the nondiabetic population (144). In Type 1 diabetes, the incidence of hypertension increases with age and the duration of DM (131), and it is almost always found concurrently with diabetic nephropathy (144).

The precise relationship between hypertension and diabetic nephropathy is not fully defined. As discussed earlier, it has been shown that hypertension often precedes the development of proteinuria by several years (5). It has been suggested that hypertension in DM is volume-dependent. In the early stages of DM, hyperglycemia leads to hyperosmolality of the extracellular fluid, so water moves into the extracellular space to maintain osmolality. Thus, a state of volume overload exists, as evidenced by the increased plasma

volume which is often noted at this stage in diabetic humans and animals (144). The resulting hypertension then contributes to the acceleration of renal damage, and declining renal function leads to impaired fluid excretion, exacerbating the plasma volume expansion and hypertension (5).

Alterations in the renin-angiotensin-aldosterone system are often noted in both hypertension and renal disease, so this system has received a considerable amount of attention. At the time diabetes is diagnosed plasma renin activity, angiotensin II, and aldosterone levels are usually normal (112). With the onset of renal disease in these patients, the activity of the renin-angiotensin-aldosterone system is normal or slightly decreased, and with advanced diabetic nephropathy circulating renin levels are often suppressed (112). It might seem that "normal" renin levels are actually inappropriately elevated for the degree of volume expansion, but absolute overproduction of renin is an uncommon cause of hypertension in DM. Simonson (144) states that several factors may be responsible for the low-renin hypertension seen in DM. The increased extracellular volume should suppress endogenous renin production. The synthesis and release of renin may also be impaired secondary to hyalinization of renal afferent arterioles or destruction of juxtaglomerular cells. In addition, there is some evidence that the cleavage of prorenin to form renin may be impaired in DM, leading to excessive release of the biologically less active renin precursor into the circulation. Renin release is partially dependent on adrenergic stimulation, so diabetic patients with neuropathy and diminished catecholamine release may have impaired renin secretion (144). Recently, it has also been suggested that elevated levels of plasma ANP in DM may be partially responsible for the decreased renin activity, since ANP has been shown to suppress plasma renin secretion (2).

Another factor that may contribute to the development of hypertension in DM is an increased sensitivity of the vasculature to pressor hormones. It has been demonstrated that the dose of angiotensin II necessary to raise the blood pressure by 20mmHg is significantly less in diabetics than controls, which suggests an enhanced vascular response in diabetics (144). Potentiation of the vasoconstrictor response to angiotensin II has also been shown in STZ-induced diabetic rats (78). Similar increased vascular responsiveness in diabetics has been noted with norepinephrine as well (144).

It has been suggested that insulin may also play a role in the pathogenesis of hypertension in DM. Hyperinsulinemia is reported to be common in Type 1 diabetics due to exogenous insulin administration (111), and insulin stimulates renal sodium reabsorption, which may predispose to volume overload (144). Total body and exchangeable sodium have been found to be increased in both experimental diabetes (2) and diabetic humans (144), but the relationship of increased exchangeable sodium to the maintenance of elevated blood pressure is unclear because it is also seen in normotensive diabetics (144). Insulin also has a stimulatory effect on the sympathetic nervous system, which causes increased release of norepinephrine (144). If the vascular sensitivity to catecholamines is increased in DM, then the combined effects of volume expansion and vasoconstriction would produce increases in blood pressure. However, hyperinsulinemia must not be the sole factor involved in causing hypertension because 50% of diabetics never develop hypertension despite having increased levels of insulin.

No single agent has been identified as the primary cause of hypertension in diabetes, which is not surprising since hypertension is not fully understood in the nondiabetic population. Its impact, however, should not be underestimated. The presence of hypertension with DM predisposes to

and accelerates the progression of coronary, peripheral, and cerebral vascular disease, as well as renal disease and other microvascular complications (131). The study of the mechanisms and control of hypertension in DM is thus a very active area of research (144).

Several of the pathophysiologic events that can occur as a result of DM have been discussed. This discussion has focused on areas which ANP and DM both affect, namely the renal and cardiovascular systems. Many complications of DM other than those addressed also exist, such as neuropathy, ocular problems, and defects in wound healing, but these will not be reviewed.

3. ANP and Diabetes Mellitus

ANP is a hormone that appears to be involved in body fluid, electrolyte, and blood pressure homeostasis, and each of these regulatory systems is affected in DM. Thus, it is not surprising that a considerable amount of attention has been focused recently on studying possible relationships between ANP and the pathophysiology of DM (Table 3). The findings of these research efforts will be briefly presented here, and they will be examined in greater detail in the discussion section.

Several studies have been conducted using rats with STZ-induced DM (2, 15, 74, 75, 86, 117, 120, 151) or alloxan-induced DM (31), and a number of studies have involved humans with Type 1 DM (14, 28, 51, 68, 92, 111, 114. 115, 126, 135, 138, 145, 147, 153, 156, 160). In the vast majority of these studies the authors have reported finding elevated levels of plasma ANP in the diabetic study groups (2, 14, 15, 31, 51, 75, 92, 111, 115, 117, 135, 138, 145, 147, 151, 153), while only a few reported finding no difference in plasma ANP

TABLE 3: Publications on ANP and diabetes mellitus.

Year	Number of Publications
1983-1984	0
1985-1986	2
1987-1988	14
1989-1990	26
Total:	42

Note: Publications identified from Medline literature search.

between diabetic and control groups (28, 74, 86, 114). In some of the investigations the elevated plasma ANP levels were found only in those diabetics with poorly controlled or untreated DM (2, 14, 15, 31, 75, 117, 151), but in other studies plasma ANP levels were also increased in diabetics treated to maintain euglycemia (51, 92, 111, 115, 138, 145, 153). Plasma volume was determined in several of the investigations, but the findings were not consistent. Jackson et al. (86) found the plasma volume to be increased in the DM group, but reported no difference in plasma ANP levels. Allen and coworkers (2) and Hebden et al. (75) reported increased plasma ANP levels in association with elevated plasma volumes in their diabetic rats, while Benigni et al. (15) found no difference in plasma volume between DM and control groups, but they did find the DM group to have increased plasma ANP levels. Interestingly, in a study of children with volume depletion secondary to diabetic ketoacidosis, plasma ANP levels were found to be decreased (156). In a few of the investigations an increased GFR (2, 117, 138), or creatinine clearance (145) was found to be positively correlated with plasma ANP levels in diabetic subjects, and Bell and coworkers (14) reported that elevated plasma ANP levels were associated with the later stages of diabetic renal disease. One study found that diabetic patients with cardiac autonomic neuropathy were more likely than those without this complication to have increased levels of plasma ANP (92). A few investigators have discovered that there are a decreased number of atrial specific granules (75, 151) or decreased level of atrial tissue ANP (31) in the atria of diabetic rats as compared to controls.

Several groups have investigated the responses of diabetic humans or rats to volume expansion, since this is the major stimulus for ANP release. All of these investigations have found that the diabetic groups have a diminished natri-

uretic response to volume expansion versus the control groups (28, 111, 114, 115, 153), and a less marked increase in plasma ANP (75, 111, 115, 153). In addition, a few investigators have examined the responses of diabetics to intravenous infusion of ANP. In the majority of these studies it was found that the diabetic groups had blunted renal responses to ANP infusion (15, 68, 120, 160), but no alterations in cardiovascular or hormonal responses (68, 160). Few conclusions as to the mechanisms responsible for these findings can be drawn, but they do make it seem likely that ANP is involved in some of the abnormalities of diabetes mellitus.

CHAPTER II

MATERIALS AND METHODS

A. EXPERIMENTAL MODEL

Numerous animal models have been used to study DM, each with certain advantages and disadvantages, and none perfectly reflecting the disease and its complications in man (59). Animals that develop Type 1 DM spontaneously include the Bio-Breeding (BB) rat (105), Chinese hamster (59), Yucatan miniature swine (124), nonobese diabetic (NOD) mouse (33), dogs (53), and several species of nonhuman primates (81). These models are useful for studying the etiology of DM and many of the metabolic and physiologic derangements that accompany it (105). The larger animals, like dogs, swine, and nonhuman primates, have longer lifespans than the rodents, making them more useful for longitudinal studies of long term complications of DM. It is also more feasible to collect repeated blood and tissue samples from the larger animals (64). The porcine (124) and nonhuman primate (81) models exhibit close cardiovascular and metabolic similarities to humans, making it more likely that analogies between species will be accurate. All of the spontaneous animal models of DM have several disadvantages though. Their availability is generally limited, and their purchase cost is high (33, 53, 59, 105, 124). In addition, the larger animals with either spontaneous or chemically induced DM (53, 81, 124) are more expensive to maintain.

Of the chemical agents that can be used to produce hyperglycemia, those that specifically destroy beta cells in the pancreas are the most useful because they more closely mimic the pancreatic lesions in Type 1 DM in humans (64). Alloxan and STZ are the two most commonly used diabetogenic

agents (73).

Alloxan is an unstable pyrimidine compound which causes massive beta cell destruction after injection (130). When high doses are used the diabetic state can be very severe and lead to death within a few weeks. Daily insulin therapy is usually required to avoid the development of diabetic ketoacidosis (130).

Streptozotocin (STZ) is a nitrosourea compound (64) that was originally used as a broad spectrum antibiotic (130). Its diabetogenic effect was first noted in 1963 (130), and STZ is now successfully used in rats, mice, hamsters, guinea pigs, dogs, and monkeys to induce DM (130). It has also been reported to be an anti-leukemic agent and a carcinogen. There is general agreement that STZ elicits a characteristic triphasic variation of blood glucose in normal, nonfasted animals. An initial, brief hyperglycemia, probably resulting from inhibition of insulin release from beta cells, occurs. Within a few hours hypoglycemia occurs as a result of massive insulin release from damaged beta cells. Permanent hyperglycemia results from insulin deficiency that appears within 24-72 hours. The effective and lethal doses of STZ are highly sensitive to age, sex, and nutritional status. The sensitivity to STZ is greatest in young, male, or fasted animals (64). There is also general agreement that STZ is a better diabetogenic agent than alloxan because it is more specific in its site of action, much less likely to cause ketoacidosis, and less prone to inter-animal variability in effective dose (73). In addition, in the rat any changes in renal morphology which may occur with STZ-induced DM appear to be the result of the diabetic state rather than to any toxic effects of the chemical (73). With alloxan, on the other hand, multiple doses or one very large dose can cause toxic renal damage (130).

Despite the fact that diabetic rodents differ from man in

certain pathophysiologic characteristics, such as failure to develop significant atherosclerosis (29), they are the major experimental animal type for studying DM (64). This is no doubt a reflection of their relatively low cost and ease of maintenance. Although rodents that develop DM spontaneously are probably a better model than STZ-induced DM, they are not as readily available as normal rats (105). Many aspects of STZ-induced DM in rats have been well-characterized, and this model is widely used (73). Thus, the STZ model was chosen for this study.

B. EXPERIMENTAL PROTOCOL

Thirty-eight male Sprague-Dawley rats with initial body weights of 230-350g were employed in this study. Toward the end of the study, eight additional male Sprague-Dawley rats were added to the normal control group. All rats were housed individually and allowed free choice access to standard rat chow and water. The rats were given one week for acclimatization before any procedures were performed.

After one week, body weights and fasting blood glucose concentrations were determined on all rats. Blood was obtained from the tail vein, and glucose concentration was measured with a Glucometer II Blood Glucose Meter (Ames Division, Miles Laboratories).

Diabetes mellitus (DM) was induced in 26 of the rats following an overnight fast by intraperitoneal (IP) injection of streptozotocin (STZ, Sigma) at a dose of 45mg STZ/kg body weight. The STZ was dissolved in a citric acid/sodium citrate buffer, 0.05M, pH 4.2, as described by Bakhle and Chelliah (10). Blood glucose levels were then checked weekly with the Glucometer II. Urine glucose was checked whenever possible with Chemstrip uGK strips (Boehringer Mannheim Diagnostics). Body weights were also monitored weekly throughout the study.

Three weeks after the induction of DM, the rats were anesthetized with 50mg sodium pentobarbital/kg body weight, IP, and placed in dorsal recumbency on a heated surgery stand. Sodium pentobarbital anesthesia was chosen because it is reported to have less effect on plasma ANP levels than other common anesthetics (54, 80). The trachea was cannulated, and the rats were ventilated with room air using a Harvard Apparatus Rodent Respirator. The common carotid artery and jugular vein were cannulated with polyethylene tubing (PE-50). After the vessels were cannulated, the rats were given 100 units of heparin via the jugular vein catheter to prevent clotting, and were allowed 15-30 minutes for stabilization before taking any measurements or blood samples.

The carotid artery catheter was connected to a Stratham physiological transducer and a Grass Model 7D polygraph to measure heart rate and arterial blood pressure.

The rats were randomly divided into several study groups, as follows.

1. Plasma and Atrial Tissue irANP Study

At the conclusion of the stabilization period, heart rate and arterial blood pressure were recorded for all of the rats (19 normal and 26 DM). The common carotid artery was used for collecting blood samples, and the rats (6 normal and 20 DM) had 0.5ml blood taken for plasma glucose determinations. Six normal and 12 DM rats then had 2.5ml of blood taken for plasma immunoreactive ANP (irANP) determination. Blood for irANP measurement was collected in chilled polypropylene tubes containing EDTA (Sigma, 1mg/ml blood) and aprotinin (Sigma, 500 KIU/ml blood). Aprotinin is a protease inhibitor obtained from bovine lung. In addition, four normal rats and seven DM rats had 0.75ml of blood taken for measurement of plasma sodium and potassium concentrations by ion selective

electrode. This was done because total body and exchangeable sodium levels have been reported to be elevated in DM (2). Sodium concentrations were corrected for hyperglycemia using the correction factor of an approximately 1.45mEq/L decrease in sodium per 100mg/dL increase in blood glucose concentration (142). During all blood sampling, heparinized saline (0.9%) was infused via the jugular vein catheter at the same rate and volume as blood was withdrawn to maintain circulatory volume, as described by Nishida (110).

After blood samples were collected, six rats in the normal group and nine rats in the DM group were sacrificed with sodium pentobarbital. The left and right atria of two rats in each group were removed, rinsed with 0.9% saline, minced, and placed in fixative containing 5.0% glutaraldehyde, 3.0% formaldehyde, and 25% picric acid in a buffer of 0.05M sodium cacodylate at pH 7.3. The tissues were kept in the fixative at 4° C until processing for electron microscopy to compare granularity between the atria of normal and DM rats. Two rats from each group had the pancreas removed and placed in Bouin's fixative prior to processing for light microscopy. The atria from four rats in the normal group and seven rats in the DM group were removed, weighed, then processed for measurement of irANP content according to the method of Tsunoda (155), as follows. The atria were boiled in 10 volumes of 0.1N acetic acid for 15 minutes. The volume of 0.1N acetic acid was then brought to 3.0ml, and the tissue was homogenized with a polytron homogenizer (Brinkmann Instruments). The samples were centrifuged for 30 minutes at 2800 rpm, 4° C in an IEC Centra 7R refrigerated centrifuge (International Equipment Company Division, Damon). The supernatant was stored at -70° C until assay.

2. Exogenous ANP Infusion Study

For this study, six rats from the normal group and six rats from the DM group were used. After cannulation of the common carotid artery and jugular vein, the bladder was exposed, emptied, and cannulated with PE-90 tubing. Fifteen minutes was allowed for stabilization, then urine was collected over time for determination of the urine flow rate and urine sodium and potassium excretion rates. Blood was then collected for the control plasma irANP and glucose measurements. The rats then received a prime of 2.5ug ANP_{3-28}/kg body weight (Bachem Inc.), followed by a continuous infusion of 0.1ug ANP_{3-28}/kg body weight for 30 minutes (50). The ANP used for the priming dose was at a concentration of 50ug/ml, and the ANP used for the infusion was at a concentration of 1.0ug/ml, so that the total volume necessary for the infusion was less than 2ml. Starting with the ANP priming dose, heart rate and arterial blood pressure were monitored continuously, and urine was collected for determination of urine flow rate and urine sodium and potassium excretion rates. Urine sodium and potassium concentrations were determined by ion selective electrodes with a DuPont Na+/K+ Analyzer. After the infusion the rats were sacrificed with sodium pentobarbital.

3. Plasma Volume Study

Since plasma volume has often been reported to be elevated in DM (2, 75, 86), 15 male Sprague-Dawley rats with initial body weights of 250-280g were used in a separate study to determine plasma volume in normal versus DM rats. In this study, rats were housed and fed as described for the previous study. Initial blood glucose concentrations were assessed using Glucostix (Ames Division, Miles Laboratories), then DM

was induced in ten of the rats with STZ, 45mg/kg, IP.

Three weeks after the induction of DM, the rats were anesthetized with 50mg sodium pentobarbital/kg body weight, IP. The trachea, jugular vein, and common carotid artery were cannulated in the same manner as described in the previous studies. To determine plasma volume, 0.05ml of 0.5% Evan's blue solution was injected via the jugular vein catheter (23). Exactly five minutes later, 2.0ml of blood was withdrawn from the carotid artery catheter. The procedure for determining plasma volume is described below. Blood was also sampled for plasma glucose measurement. The rats were then sacrificed with sodium pentobarbital.

C. BIOCHEMICAL ASSAY PROCEDURES

1. Plasma Glucose Assay

Blood samples for the glucose assay were centrifuged for ten minutes at 4000rpm in an Eppendorf Centrifuge. Plasma was then stored at -20° C until assay. Plasma glucose was determined with the aid of Kit 16-11 and 315-100 from Sigma Diagnostics. Briefly, standards were prepared with glucose concentrations of 50mg/dL, 100mg/dL, 150mg/dL, 200mg/dL, 300mg/dL, 400mg/dL, and 500mg/dL. 10ul aliquots of each standard and sample in duplicate were added to 3.0ml of Glucose (Trinder) reagent and absorbance read at 505nm on a Cary 219 spectrophotometer (Varian) at timed intervals. The Glucose (Trinder) reagent contains the reagents and enzymes necessary to produce a color whose absorbance at 505nm is directly proportional to the glucose concentration of the sample. The reaction is:

$$\text{Glucose} + H_2O + O_2 \xrightarrow{\text{*Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

H_2O_2 + *4-Aminoantipyrine + *p-Hydroxybenzene sulfonate

*peroxidase
----------> Quinoneimine dye + H_2O

Those compounds marked by an * are contained in the Glucose (Trinder) reagent.

The glucose concentrations of the samples were determined using the standard curve generated from linear regression analysis of the standard concentrations versus their absorbances.

2. Plasma and Atrial Tissue ANP Assay

Blood samples were centrifuged at 4° C, 2800rpm for 30 minutes in an IEC Centra-7R centrifuge. Plasma was stored at -70° C until assay. Immediately prior to the assay procedure, the plasma samples were extracted using a protocol recommended by Peninsula Laboratories, Inc. Extraction is necessary to eliminate nonspecific interference of plasma proteins and to concentrate the plasma (65). Plasma proteins damage radioiodinated ligand (26). Samples were first acidified with 0.1% trifluoroacetic acid (TFA), then applied to Sep-Pak C18 cartridges (Waters Associates) which had been activated with 0.1% TFA and 60% acetonitrile in 0.1% TFA. ANP was then eluted from the cartridges with 60% acetonitrile in 0.1% TFA, and the eluant was evaporated to dryness in a SpeedVac Concentrator (Savant). The residues were then dissolved in assay buffer supplied by Peninsula Laboratories.

The atrial tissue extracts were centrifuged a second time at 2800rpm, 4° C for 30 minutes in an IEC Centra-7R centrifuge. The supernatant was then used in the assay.

The assay employed a radioimmunoassay (RIA) kit from Peninsula Laboratories for alpha-rat ANP. The assay is based on competition between (^{125}I)-labelled rat ANP and rat ANP in

the samples for binding sites to a limited amount of antibodies specific for rat ANP. As the amount of standard or sample ANP increases, the amount of (^{125}I) rat ANP able to bind to the antibody is decreased. Separation of bound ANP from unbound ligand is achieved by precipitation and centrifugation of the antibody bound fraction. By measuring the radioactivity of the precipitate, a standard curve is constructed and concentration of ANP in the samples is interpolated from this curve. The antibody used in this assay is rabbit antiserum specific for rat ANP and goat anti-rabbit IgG serum as the second antibody. The standards were made at concentrations ranging from 1-128pg ANP/tube, and all samples were assayed in duplicate. Radioactivity of the pellets was measured in a Beckman Gamma 5500 counter. Extraction efficiency was 40%, intraassay variation was 7%, and interassay variation was 17%. These coefficients of variation are based on two assay runs. The specificity of this RIA for rat ANP has been reported by Peninsula Laboratories (Table 4). Virtually all data on ANP concentrations have been obtained using RIA's, and greater than 50% of the studies reported in the literature have used rabbit ANP antiserum from Peninsula Laboratories (26).

It should be noted that at one point during the course of the study an RIA kit from Amersham for rat atriopeptin III was used. It seemed to give satisfactory results, though plasma irANP levels were much higher and tissue irANP levels were lower than those obtained with the RIA from Peninsula. The manufacture of the kit from Amersham was discontinued during the middle of this study, however. Only results obtained with the RIA from Peninsula are reported.

TABLE 4: Specificity of RIA.

SPECIFICITY

PEPTIDE	% CROSS REACTIVITY
Rat ANP (1-28)	100
Rat ANP (5-28)	100
ANP (8-33)	90
ANP (18-28)	57
Rat ANP (13-28)	50
Gamma ANP	40
Rat ANP (5-27)	27
Rat ANP (5-25)	3
Somatostatin	0
Oxytocin	0
Arginine vasopressin	0
Brain natriuretic peptide (porcine)	0

Note: This table is adapted from the Peninsula Laboratories, Inc., product brochure for alpha rat atrial natriuretic polypeptide (Catalog No. RIK9103).

3. Plasma Volume Determination

Plasma volume was determined as described by Bruckner-Kardoss and Wastmann (23). The blood samples were centrifuged for 30 minutes at 2800rpm in an IEC Centra-7R centrifuge. 0.5ml of the stained plasma was then mixed with 5.5ml of 0.9% saline. Standards were prepared by adding 0.05ml Evan's blue solution to 6, 9, 12, 14, and 16ml of 0.9% saline. Then 0.5ml of these dilutions were added to 5.0ml of 0.9% saline plus 0.5ml normal rat plasma. Absorbance of the solutions at 610nm was read in a Cary 219 spectrophotometer, and all standards and samples were measured in duplicate. Plasma volume was determined using the standard curve generated using linear regression analysis of absorbance versus standard volumes.

D. MORPHOLOGICAL STUDIES

1. Histology of the Pancreas

After fixing in Bouin's fluid, the tissue blocks were routinely dehydrated with increasing concentrations of ethanol, cleared with increasing concentrations of xylene, embedded in paraffin, and sectioned. The mounted sections were then deparaffinized with xylene, then decreasing strengths of ethanol, and water. The sections were stained with Gomori's Aldehyde Fuchsin combined with Masson tri-chrome (162). Briefly, the sections were placed in Lugol's solution for 15 minutes, washed, cleared in alcohol ammonia, washed, rinsed in 70% ethanol, then placed in aldehyde fuchsin for 4-24 hours to stain the beta cell granules. They were then rinsed in two changes of 70% ethanol and washed. Alpha cell granules were stained with Ponceau acid fuchsin for five minutes. The slides were rinsed, placed in 10% phospholybdic acid for five minutes, rinsed, then stained with light green

solution for five minutes to stain the delta cell granules. Finally, the sections were rinsed in 0.5% acetic acid for two minutes, dehydrated in ethanols, and cleared with xylenes. This procedure results in differential staining of the granules, such that alpha cell granules are red, beta cell granules are blue, and delta cell granules are green.

2. Electron Microscopy of the Atria

The fixed tissue was washed in two changes of cold 0.1M sodium cacodylate buffer, then post-fixed in 1.0% osmium tetroxide solution buffered with 0.1M sodium cacodylate for two hours. The tissues were then washed again in 0.1M sodium cacodylate buffer, dehydrated through an ethanol series, and cleared with propylene oxide (PO). The tissue was then infiltrated in a 1:1 resin:PO mixture overnight. The following day, the tissues were infiltrated with a 3:1 resin:PO mixture for 4-6 hours and transferred to pure resin for two hours before placing in a curing oven at 60° C for approximately 48 hours. The resin used was Polybed 812.

After curing, the blocks were hand-trimmed, and thin sections approximately 90-120nm thick were cut using a Reichert-Jung Ultracut E ultramicrotome. The sections were decompressed with chloroform and collected on 3.0mm copper mesh grids.

The sections on the grids were stained with 2.0% uranyl acetate for 12 minutes, then rinsed and stained with lead citrate for five minutes.

Following staining, the sections were examined in a JEOL 100CX-II Scanning Transmission Electron Microscope, and micrographs were taken and developed in routine fashion.

E. STATISTICAL ANALYSIS

All results are presented as mean values ± SE. The statistical significance of the parameters studied was determined with a student's t test. The results were considered statistically significant at $p < 0.05$ for all tests (95).

CHAPTER III
RESULTS

A. CHARACTERISTICS OF THE STZ-DIABETIC RAT MODEL

Figure 7 represents summary data of the mean initial body weights and final body weights on the day of the experiment in the normal and DM groups. As expected, the normal group had gained an average of 124.0 ± 12.1(SE)g, while the DM group had gained an average of only 39.4 ± 8.2(SE)g. The difference between the two groups was highly significant ($p < 0.001$). The net change in body weight was measured over 33.5 ± 3.4(SE) days in the normal group, compared with 38.7 ± 2.3(SE) days in the DM group. This difference was not statistically significant. It should be noted that initial body weights between the two groups were very similar. The mean initial body weights of the normal and diabetic groups were 301.5 ± 13.5(SE)g and 285.9 ± 8.3(SE)g, respectively. This did not represent a statistically significant difference in initial body weight.

The initial measurements of blood glucose with the Glucometer II, before the induction of diabetes, were also very similar between the two groups, with a mean value of 85.2 ± 5.5(SE)mg/dL in the normal group and 86.1 ± 4.9(SE)mg/dL in the DM group (Figure 8). At the conclusion of the study, on the day of the experiment, the normal group had a mean plasma glucose value of 131.5 ± 9.3(SE)mg/dL (n=12), as determined by colorimetric assay, whereas the DM group had a mean plasma glucose value of 440.6 ± 25.1(SE)mg/dL (n=26) ($p< 0.0001$) (Figure 9). In addition, whenever it was possible to check urine glucose, the DM rats tested positive for glucosuria, while the normal rats were consistently negative for glucosuria.

Subjectively, histology of the pancreas in two rats from each group appeared to show a decrease in the number of blue-staining beta cell granules in the islets of the DM group when compared to the normal group (Figure 10).

Similar results regarding body weight and plasma glucose were found in the separate group used to study plasma volume. In these rats, the normal group gained an average of 140.4 ± 11.1(SE)g body weight (n=5) over 25.4 ± 0.9(SE) days, while the DM group gained an average of 87.7 ± 4.4(SE)g body weight (n=10) over 28.1 ± 1.8(SE) days ($p < 0.001$). Mean plasma glucose at the end of the study was 140.6 ± 16.3(SE)mg/dL (n=5) in the normal group and 496.6 ± 32.0(SE)mg/dL (n=10) in the DM group ($p < 0.001$). The plasma volume was significantly increased in the DM group at 4.43 ± 0.12(SE)ml/100g body weight (n=9) versus 3.79 ± 0.06(SE)ml/100g body weight in the normal group (n=4) ($p<0.01$) (Figure 11). One rat in each group experienced a marked blood loss during catheterization of the carotid artery, so these rats were excluded from the calculations of plasma volume.

B. COMPARISON OF PLASMA AND ATRIAL TISSUE irANP MEASUREMENTS IN DIABETIC AND NORMAL RATS

Data from 12 normal rats and 18 STZ-induced diabetic rats are summarized in Figure 12. The average plasma irANP of the normal rats was 86.3 ± 12.9(SE)pg/ml. In contrast, the mean value of plasma irANP of diabetic rats was 149.6 ± 19.4(SE)pg/ml, or about twice the normal rat values. The difference between the two groups was highly significant ($p < 0.01$). Atrial tissue irANP levels in rats killed after the control period were 60.15 ± 1.3(SE)ng/mg atrial weight (n=4) in the normal group. This may be compared with the mean value of 38.07 ± 7.8(SE)ng/mg atrial weight (n=7) in rats with DM (Figure 13). This difference was highly significant between

the two groups ($p<0.02$). In the electronmicrographs of the rat atria it subjectively appeared that the atrial myocytes in the normal rats had a greater density of atrial granules than the myocytes in the DM rats. This observation is consistent with the biochemical measurements of atrial tissue irANP reported above. Structurally, the atrial tissue appeared similar in the micrographs from the diabetic and normal rat atria.

C. HEMODYNAMIC AND PLASMA ELECTROLYTE FINDINGS

Table 5 depicts the mean heart rate and mean arterial blood pressure for the two groups during the control period. Mean heart rate in the normal group was 403.2 $\pm$ 15.3(SE)beats/minute (n=19) and 381.3 $\pm$ 10.8(SE)beats/minute (n=26) in the DM group. The normal group had an average mean arterial blood pressure (MABP) of 121.2 $\pm$ 6.2(SE)mmHg, and the DM group had an average MABP of 132.1 $\pm$ 6.9(SE)mmHg. These differences were not significant.

Plasma sodium and potassium values are presented in Table 6. The plasma sodium concentration was 149.7 $\pm$ 2.4(SE)uEq/L in the normal group (n=4) and 148.3 $\pm$ 4.6(SE)uEq/L in the DM group (n=7). These values have been corrected to account for the degree of hyperglycemia. The plasma potassium concentration was 3.8 $\pm$ 0.3(SE)uEq/L in the normal group and 3.9 $\pm$ 0.2(SE)uEq/L in the DM group. There was no significant difference in these electrolyte values between the two groups.

D. RESPONSE TO ANP INFUSION

Six rats from the normal group and six rats from the DM group were used to study the effects of ANP_{3-28} infusion on heart rate, MABP, urine flow, and urine sodium and potassium excretion rates.

Mean urine flow rate during the control period was 3.6 ± 0.6(SE)ul/min in the normal group, and it increased significantly to 20.6 ± 5.4(SE)ul/min during the ANP infusion ($p<0.02$) (Figure 14). In the DM group the mean urine flow rate during the control period was 30.7 ± 16.0(SE)ul/min and 36.2 ± 11.0(SE)ul/min during ANP infusion (Figure 15). This did not represent a significant change in urine flow rate in the DM group. The difference in basal urine flow rates between the DM and normal groups was not statistically significant.

The urine sodium excretion rate during the control period in the normal group was 0.3 ± 0.1(SE)uEq/min and during ANP infusion it increased significantly to 2.5 ± 1.0(SE)uEq/min ($p<0.05$) (Figure 16). In the DM group the urine sodium excretion rate was 1.1 ± 0.7(SE)uEq/min during the control period, and it rose to 2.1 ± 0.7(SE)uEq/min during the ANP infusion. This was not a significant increase (Figure 19). The difference in basal urine sodium excretion rates between the two groups was not statistically significant.

During the control period, the urine potassium excretion rate for the normal group was 0.5 ± 0.2(SE)uEq/min and it showed a significant increase to 1.4 ± 0.4(SE)uEq/min during ANP infusion ($p<0.05$) (Figure 18). The urine potassium excretion rate was 2.2 ± 1.0(SE)uEq/min during the control period in the DM group, and it was 2.0 ± 0.4(SE)uEq/min during ANP infusion. This difference was not statistically significant (Figure 19). The difference in basal urine potassium excretion rates between the two groups was not statistically significant.

Figure 20 shows the changes in heart rate observed during the infusion. There were no significant changes in heart rate within or between the two groups. Figure 21 shows the changes in MABP observed during the infusion. Although the MABP dropped from the control level within the first 90 seconds of

infusion in both groups, the decrease was not significant, and the MABP gradually increased over the course of the infusion to approximately the same value as before the infusion.

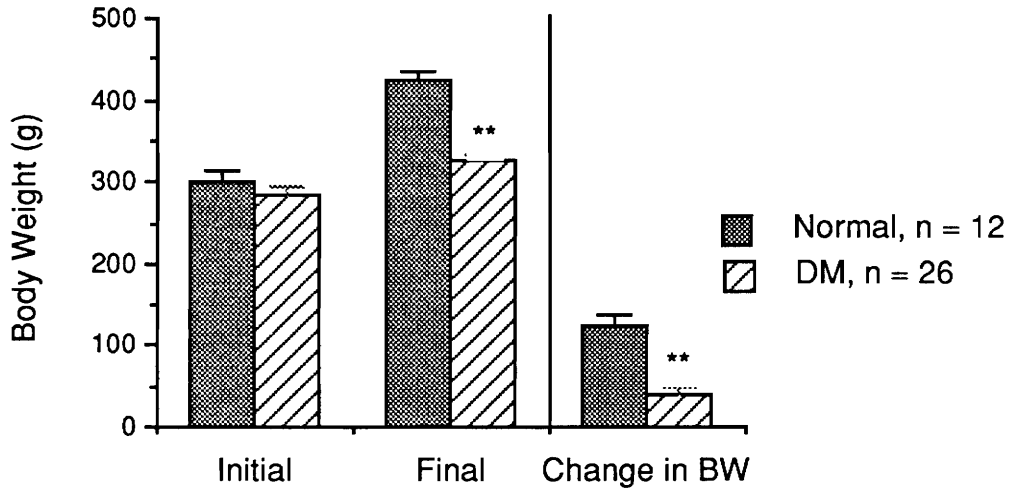

FIGURE 7: Body weight. Initial body weight represents body weight (g) at the beginning of the study, final body weight represents body weight (g) at the conclusion of the study, and change in body weight represents the net change in body weight (g) over the course of the study. The net change in body weight was measured over 33.5 $\pm$ 3.4 (SE) days for the normal group and 38.7 $\pm$ 2.3 (SE) days for the diabetic group. DM = diabetes mellitus group. n = number of animals in the group. The vertical brackets represent the standard error of the mean. **p<0.0001 for comparison between normal and diabetic groups.

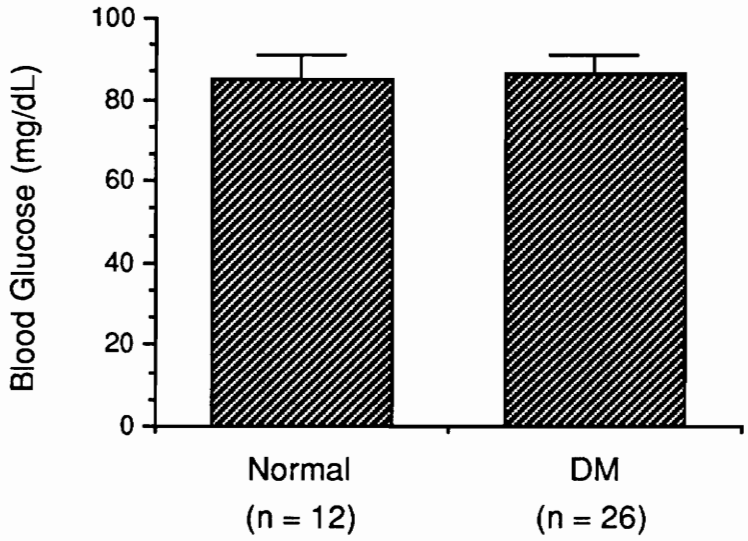

FIGURE 8: Initial blood glucose. Values represent mean blood glucose concentrations (mg/dL) at the beginning of the study. DM = diabetes mellitus group. n = number of animals in the group. Brackets represent the standard error of the mean. There is no significant difference between the two groups.

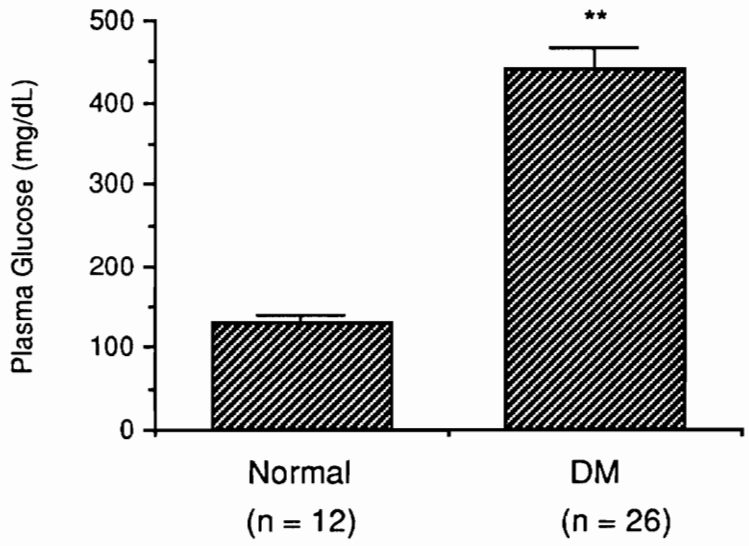

FIGURE 9: Final plasma glucose. Values represent mean plasma glucose concentrations (mg/dL) at the conclusion of the study. DM = diabetes mellitus group. n = number of animals in the group. Brackets represent the standard error of the mean. **p<0.0001 for comparison between normal and diabetic groups.

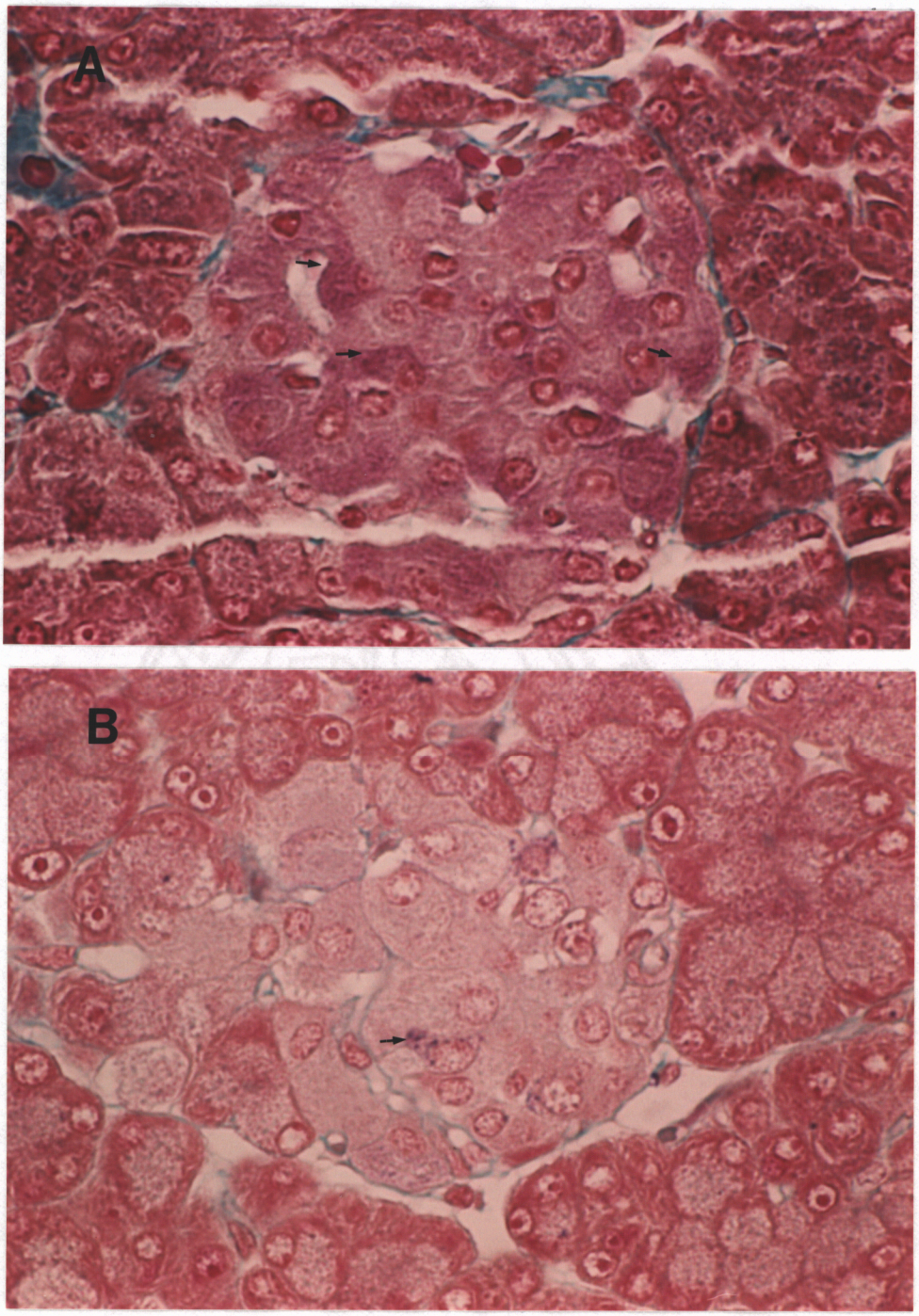

FIGURE 10: Photomicrographs of pancreatic tissue. A) photomicrograph of islet of Langerhans from a rat in the normal group. Note numerous blue-staining granules in beta cells (→). B) photomicrograph of islet of Langerhans from a rat in the diabetic group. Note marked decrease in the number of blue-staining granules in beta cells (→). Magnification x200.

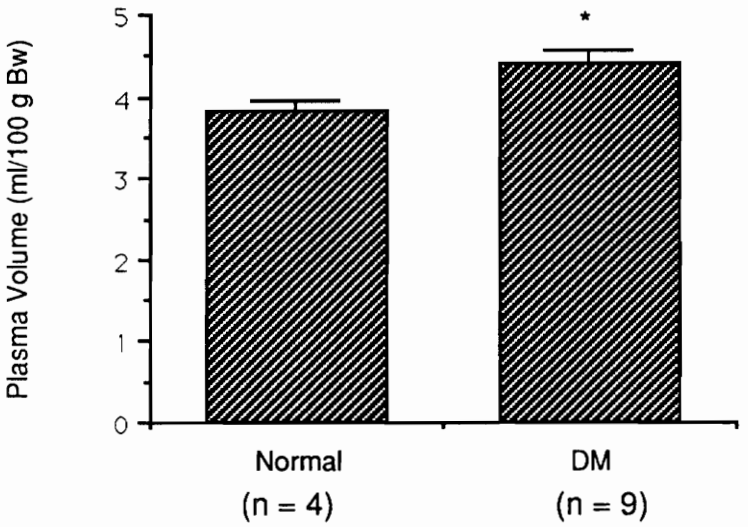

FIGURE 11: Plasma volume. Values represent the plasma volume in the Plasma Volume study group, expressed as ml plasma per 100 g body weight. DM = diabetes mellitus group. n = number of animals in the group. *$p < 0.01$.

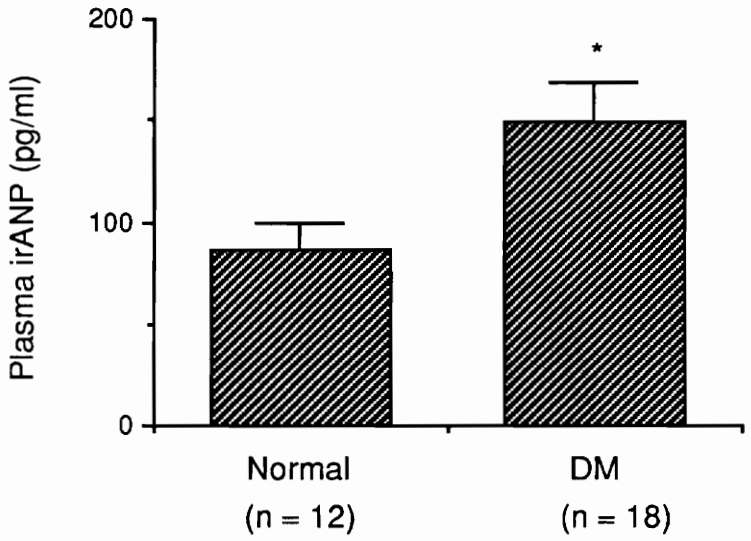

FIGURE 12: Plasma irANP. Values represent mean plasma irANP (pg/ml) in the normal and diabetic groups. DM = diabetes mellitus group. n = number of animals in the group. Brackets represent the standard error of the mean. *$p<0.01$ for comparison between the normal and diabetic groups.

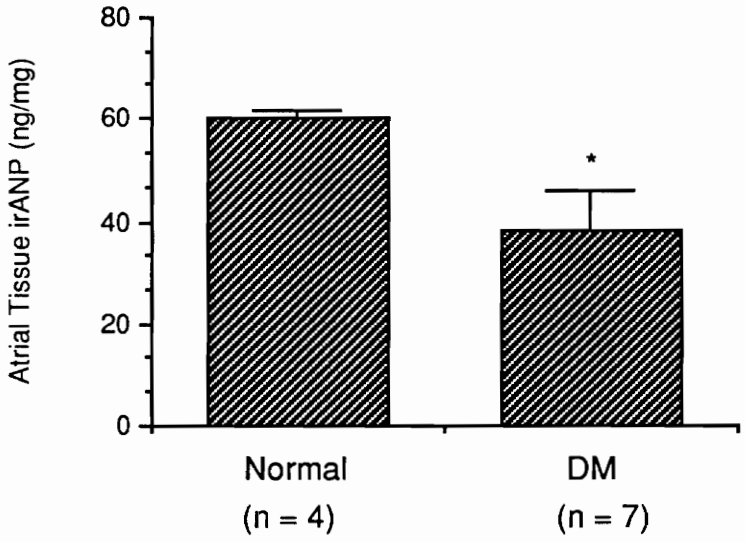

FIGURE 13: Atrial tissue irANP. Values represent mean atrial tissue irANP (ng/mg) levels in the normal and diabetic groups. DM = diabetes mellitus group. n = number of animals in the group. *$p<0.02$ for comparison between the normal and diabetic groups.

TABLE 5: Heart rate and mean arterial blood pressure.

Group	HR (bpm)	MABP (mmHg)
Normal (n = 19)	403.2 ± 15.3	121.2 ± 6.2
DM (n = 26)	381.3 ± 10.8	132.1 ± 6.9

Note: Values are mean ± SE. DM = diabetes mellitus group. HR = heart rate. bpm = beats/min. MABP = mean arterial blood pressure. n = number of animals in the group.

TABLE 6: Plasma sodium and potassium concentrations.

Group	Plasma Concentration (mEq/L)	
	Sodium	Potassium
Normal (n = 4)	149.7 ± 2.4	3.8 ± 0.3
DM (n = 7)	148.3 ± 4.6	3.9 ± 0.2

Note: Values are mean ± SE. DM = diabetes mellitus group. n = number of animals in the group. The sodium values have been corrected for the degree of hyperglycemia (see text for details).

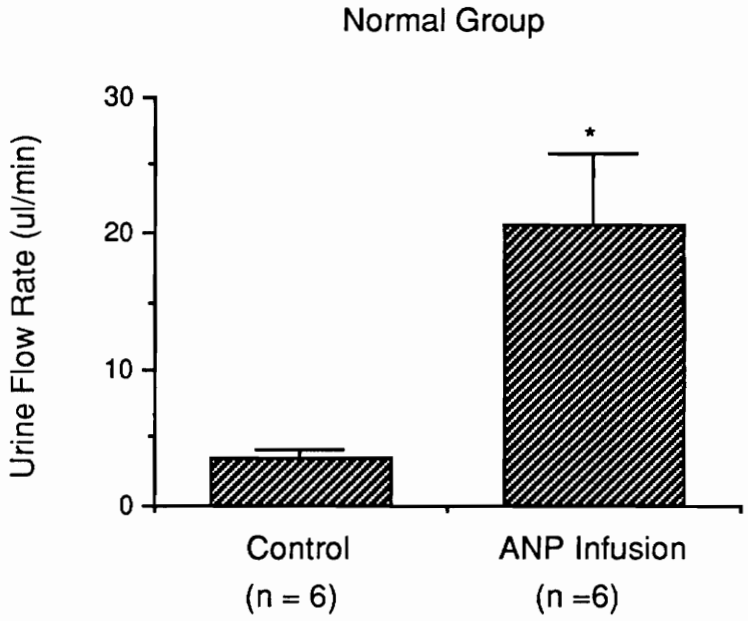

FIGURE 14: Urine flow rates in the normal group. Values represent the mean urine flow rates (ul/min) during the control period and during intravenous infusion of ANP (2.5 ug/kg body weight prime followed by 0.1 ug/kg/min for 30 minutes). n = number of animals in the group. Brackets represent the standard error of the mean. *p<0.02.

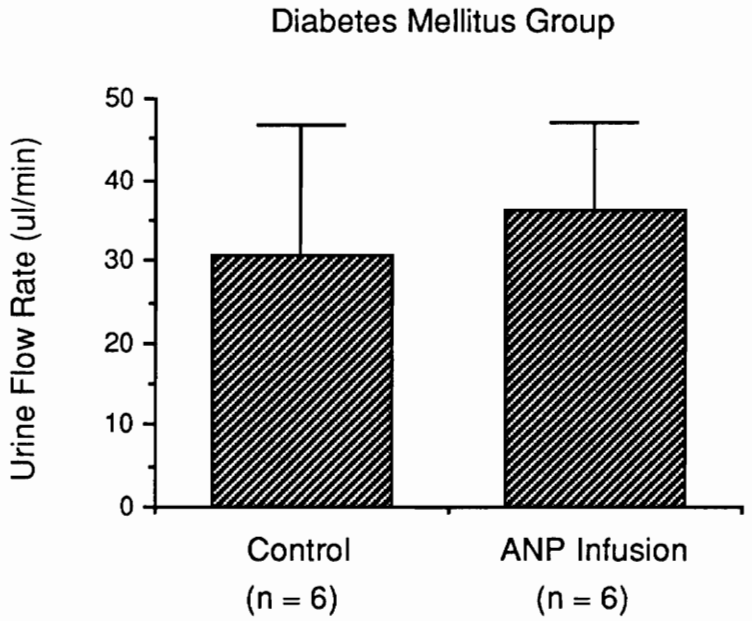

FIGURE 15: Urine flow rates in the diabetes mellitus group. Values represent mean urine flow rates (ul/min) during the control period and during intravenous infusion of ANP (2.5 ug/kg body weight prime followed by 0.1 ug/kg/min for 30 minutes). n = number of animals in the group. Brackets represent the standard error of the mean.

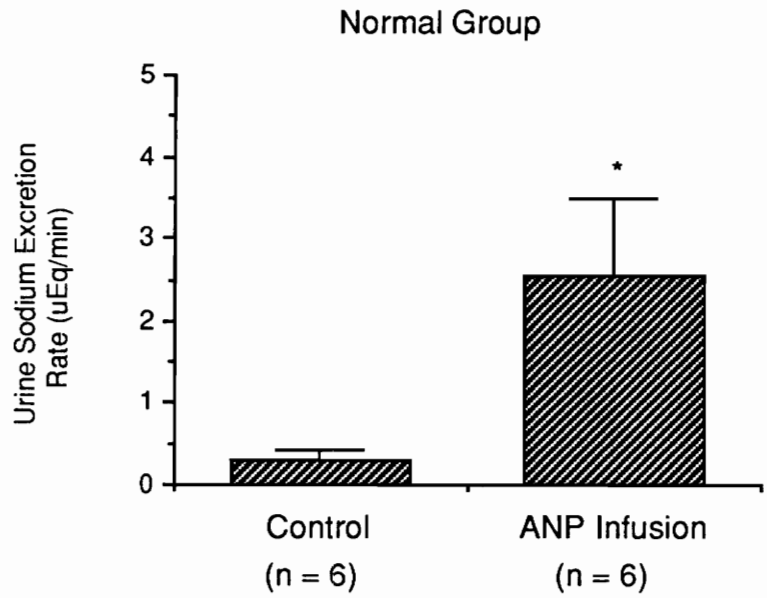

FIGURE 16: Urine sodium excretion rates in the normal group. Values represent the mean urine sodium excretion rate (uEq/min) during the control period and during intravenous infusion of ANP (2.5 ug/kg body weight prime followed by 0.1 ug/kg/min for 30 minutes). n = number of animals in the group. Brackets represent the standard error of the mean. *$p<0.05$.

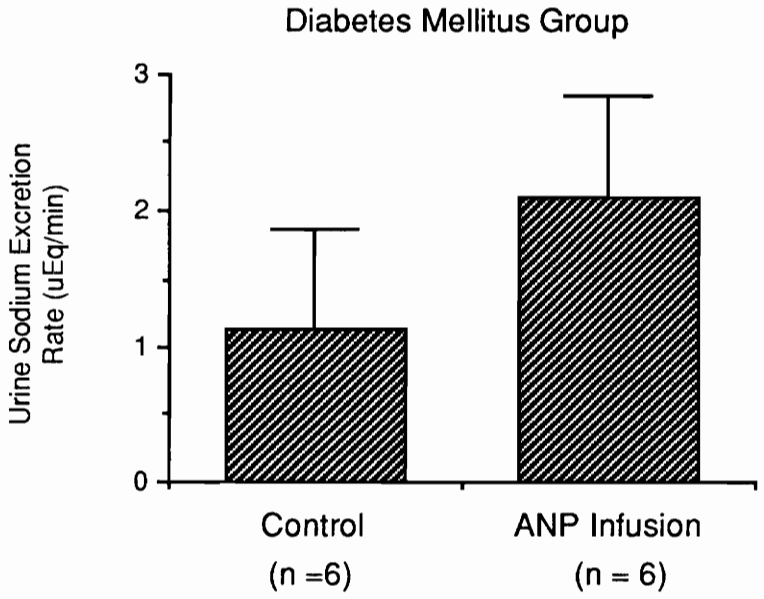

FIGURE 17: Urine sodium excretion rates in the diabetes mellitus group. Values represent the mean urine sodium excretion rate (uEq/min) during the control period and during intravenous infusion of ANP (2.5 ug/kg body weight prime followed by 0.1 ug/kg/min for 30 minutes). n = number of animals in the group. Brackets represent the standard error of the mean.

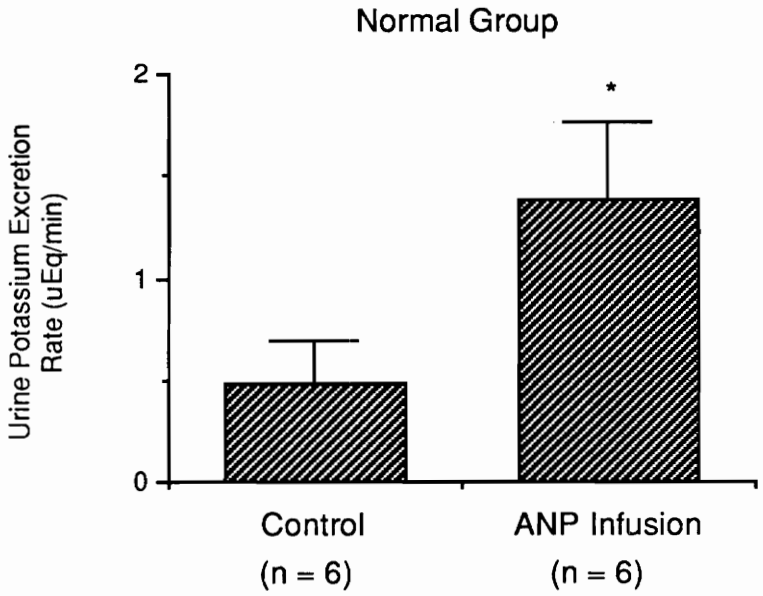

FIGURE 18: Urine potassium excretion rates in the normal group. Values represent the mean urine potassium excretion rate (uEq/min) during the control period and during intravenous infusion of ANP (2.5 ug/kg body weight prime followed by 0.1 ug/kg/min for 30 minutes). n = number of animals in the group. Brackets represent the standard error of the mean. *$p<0.05$.

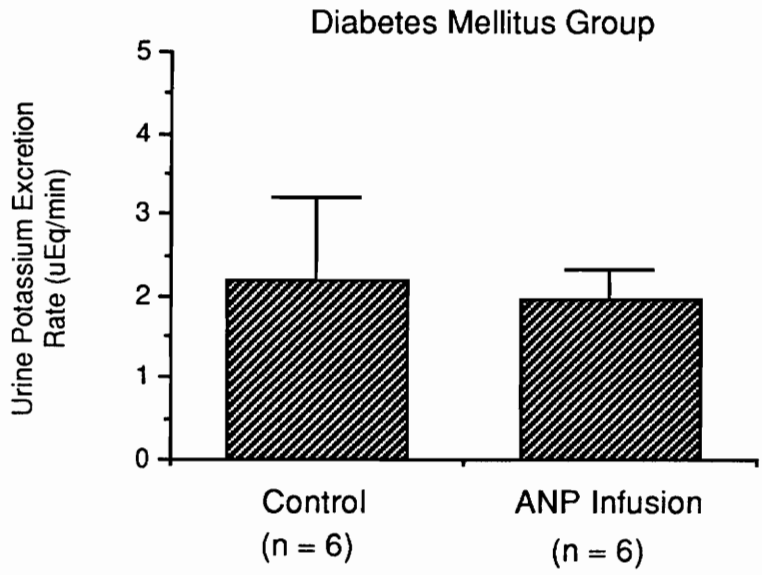

FIGURE 19: Urine potassium excretion rates in the diabetes mellitus group. Values represent the mean potassium excretion rate (uEq/min) during the control period and during intravenous infusion of ANP (2.5 ug/kg body weight prime followed by 0.1 ug/kg/min for 30 minutes). n = number of animals in the group. Brackets represent the standard error of the mean.

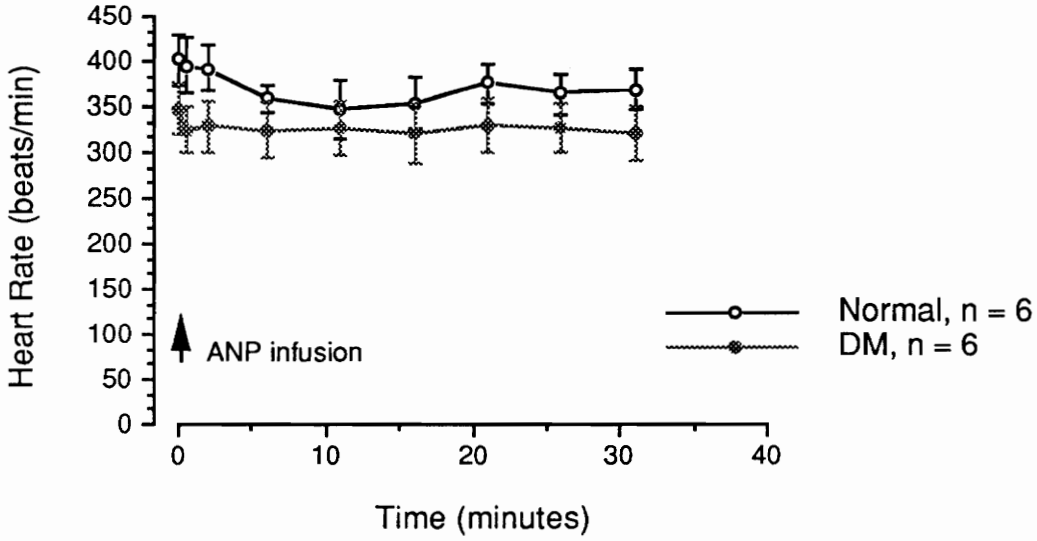

FIGURE 20: Heart rate during ANP infusion. Values represent mean heart rates during intravenous infusion of ANP (2.5 ug/kg body weight prime followed by 0.1 ug/kg/min for 30 minutes). DM = diabetes mellitus group. n = number of animals in the group. Brackets represent the standard error of the mean.

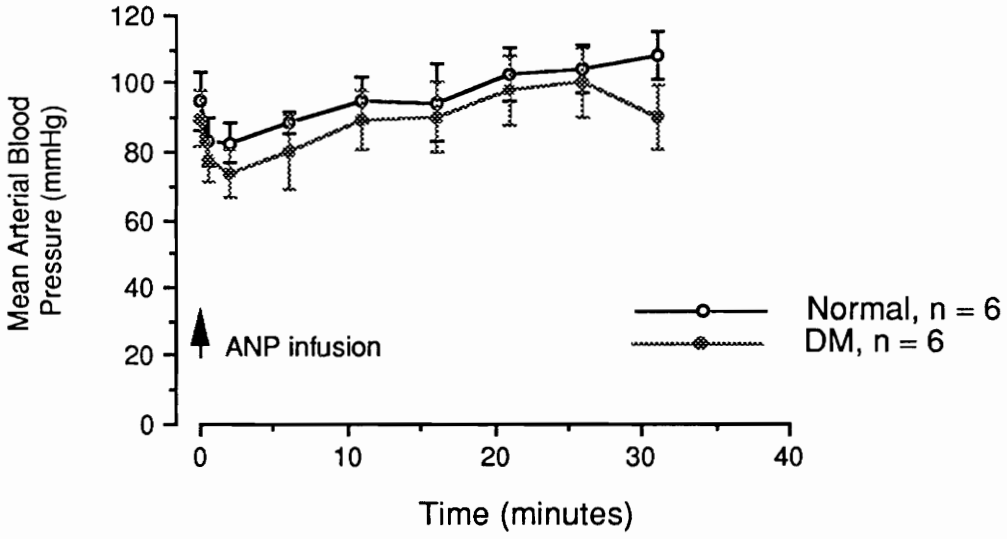

FIGURE 21: Mean arterial blood pressure during ANP infusion. Values represent mean arterial blood pressure during the intravenous infusion of ANP (2.5 ug/kg body weight prime followed by 0.1 ug/kg/min for 30 minutes). DM = diabetes mellitus group. n = number of animals in the group. Brackets represent the standard error of the mean.

CHAPTER IV

DISCUSSION

Diabetes mellitus is a disease characterized by alterations in body fluid balance, cardiovascular function, renal function, and many other body functions. These alterations involve systems in which ANP is also thought to be intimately involved, so it was of interest to explore possible relationships between ANP and DM in the STZ-induced diabetic rat model. This was done by examining plasma and atrial tissue levels of immunoreactive ANP and renal response to ANP infusion in STZ-induced diabetic and nondiabetic rats.

Before characterizing the findings related to ANP, it is important to verify the diabetic animal model of this study. Concurring with the established characteristics of diabetic animals (2, 73, 75, 127), this study shows that the intraperitoneal injection of STZ causes persistent hyperglycemia (Figure 9) and glucosuria in rats. In addition, the STZ-treated group showed a significantly diminished weight gain over the study period compared to the untreated group (Figure 7). This effect is likely due to the lack of circulating insulin and its anabolic actions and the loss of glucose in the urine (127). Histologic examination of pancreatic tissue from STZ-treated and untreated rats further indicated that the presence of diabetes in the STZ-treated group was of pancreatic origin. There was a noticeable lack of blue-staining granules in the vast majority of beta cells in the islets of Langerhans from the STZ-treated rat pancreatic tissue as compared to the untreated rat pancreatic tissue, though this was a subjective evaluation (Figure 10). The lack of granularity in the beta cells of the STZ-treated group indicates a lack of insulin in these cells (165), and lack of insulin in the beta cells results in insulinopenia, which is

the hallmark of Type 1 DM (127).

This study confirms earlier investigations (15, 117) showing that short term diabetes has no significant effect on basal cardiovascular function (Table 5). Tahiliani et al. (148) showed that STZ-induced diabetes in rats did not result in alterations in myocardial function until 6 weeks post-diabetic induction. Hebden et al. (75) and Todd et al. (151) reported that their diabetic rats exhibited significant hypotension and bradycardia when compared to control rats. Their studies were more chronic than this investigation and were carried out in conscious animals, so perhaps the bradycardia and hypotension were a reflection of cardiovascular abnormalities that develop with diabetes of longer duration or that are more apparent in the conscious state. Differences in the severity of the metabolic states may also contribute to the discrepancies between these studies.

Plasma volume was found to be significantly increased in the diabetic group as compared to the normal group in this investigation (Figure 11). Allen and coworkers (2) reported finding an increased plasma volume in STZ-induced diabetic rats in association with an increase in exchangeable sodium. They also found a strong positive correlation between plasma glucose concentration and exchangeable sodium, and stated that hyperglycemia would be expected to increase proximal tubular reabsorption of glucose and sodium, both of which are transported together in a 1:1 molar ratio in the proximal tubule (2). Exchangeable sodium was not measured in this study, but it has been consistently reported to be elevated in diabetic animals (2, 86) and humans (144, 153), so it may have been a contributing factor to the increased plasma volume in the DM group found in this study. Hebden et al. (75) reported finding an increased blood volume in diabetic rats, but they found no difference in right atrial pressure between the diabetic and normal groups. This led them to speculate that

differences in body composition between normal and diabetic animals may account for the apparent volume expansion in the diabetic animals, but that it may not represent a true volume expansion. Right atrial pressure was not measured in this study.

In this study we have found that acute STZ-induced diabetes in the rat produced no significant change in basal plasma sodium and potassium concentration (Table 6). Similar findings have also been reported in studies on rats (117) and humans (153, 163). In Weidmann's study (163) plasma sodium and total body exchangeable sodium were measured concurrently. They found no difference in plasma sodium concentrations between normal and diabetic individuals, but they did find that the diabetics had a significantly increased exchangeable sodium concentration. Thus it would appear that plasma sodium is not a reliable indicator of total body sodium.

The baseline urine flow rates and urine sodium and potassium excretion rates corresponded closely with values reported in the literature for both normal (2, 50, 73, 120) and STZ-induced diabetic rats (73, 117). The higher urine flow rate found in the DM group as compared to the normal group during the control period is a common finding (2, 73) and may reflect an increased GFR in the DM group, in addition to osmotic diuresis secondary to hyperglycemia. GFR was not measured in this study, but it has frequently been reported to be elevated in diabetic rats (15, 117). Greater urinary sodium and potassium excretion rates under baseline conditions in DM versus control rats have also been reported, and this increased electrolyte excretion rate has been found to correlate with osmotic diuresis, increased GFR, and increased electrolyte intake in diabetic rats as a result of hyperphagia (73).

Most importantly, the results of the present study have demonstrated that plasma irANP levels were significantly

elevated in the DM group as compared to the normal group (Figure 12) and that the atrial tissue irANP levels were significantly decreased in the DM group (Figure 13). In addition, renal responses to intravenous ANP infusion were significantly blunted in the DM group versus the normal group as determined by changes in urine flow rate and urine sodium and potassium excretion rates (Figures 14-19). As discussed in the literature review, there have been a number of studies which found elevated plasma levels of ANP in diabetic rats and humans. Numerous theories have also been suggested to explain these changes (75, 151). Nevertheless, an increased plasma concentration of any endogenous compound must be due either to increased release or decreased clearance of the substance, or a combination of the two (151).

Several possibilities must be considered to explain the observed ANP changes in diabetes. Ortola et al. (117), Allen et al. (2), and Hebden et al. (75) all mention the possibility that increased plasma volume in diabetes could provide the stimulus for increased ANP release from the atria. It is interesting to note that in this study we found that the DM group had a significantly greater plasma volume than the normal group on a body weight basis (Figure 11). These observations are consistent with other reports (2, 75, 86). In contrast, Benigni et al. (15) reported no significant difference in plasma volume between STZ-induced diabetic rats and nondiabetic rats, though the diabetic rats had increased plasma ANP levels. They also cited a number of contradictory reports in the literature involving both diabetic rats and humans. There are no clear explanations to account for these discrepancies. Differences in glycemic control and hydration status at the time of plasma volume measurement are likely influential factors.

There is also considerable evidence supporting a positive correlation between degree of metabolic control, plasma

glucose concentrations, and plasma ANP levels in diabetes (14, 15, 117). Poor metabolic control would lead to an elevated plasma volume through renal reabsorption of sodium and water with the increased filtered load of glucose, and this in turn would lead to stimulation of ANP release (14). Interestingly, Gibbs (60) found that ANP release by dispersed atrial cells in vitro was increased in response to perfusion with media made hypertonic with glucose, which suggests that hyperglycemia itself may be responsible for increased plasma ANP levels in DM. The significance of this finding and functional existence of this effect in normal physiological conditions is unclear and has yet to be established (75).

It has been suggested that there may me an interaction between the elevated plasma ANP levels in diabetics and renal changes in DM. In a study by Sawicki et al. (138), Type 1 diabetic patients with stages of nephropathy ranging from glomerular hyperfiltration to overt nephropathy had elevated plasma ANP levels when compared to non-nephropathic diabetic patients and control subjects. Similar findings were reported by Suzuki et al. (147). Sawicki's group found no evidence of volume overload in these patients and speculated that the increased ANP concentrations are due to renal pathophysiology and that ANP secretion may be stimulated by intrarenal hypertension. This may be a factor early in DM with glomerular hyperfiltration. Bell et al. (14) found a positive correlation between plasma ANP level and microalbuminuria in diabetic patients. They hypothesized that increased ANP secretion in hyperglycemic patients may be involved in the early hemodynamic changes in DM and may cause increased glomerular capillary permeability leading to microalbuminuria. It also seems possible that since the kidneys are a major clearance organ for ANP (79), the elevated plasma ANP levels seen in overt nephropathy with diminished GFR may be due to decreased clearance of the peptide.

Other factors, such as stress, may also influence plasma ANP levels. Hebden and coworkers (75) suggested that the elevated plasma ANP levels in STZ-induced diabetic rats are related to stress. They have found that STZ-treated animals are less able to handle surgical intervention and react more strongly to external stimuli, such as noise, than control animals. There is evidence that ANP release is stimulated by acute stress (19, 80), so this could have been a factor in this investigation if the DM rats were more stressed by the experiment. However, elevated plasma ANP levels in DM have been noted under a variety of experimental conditions in both rats and humans, and stress levels were probably quite variable in these situations.

One final point concerning increased plasma ANP levels in DM that may be worth considering is the finding by Uehlinger et al. (157) that ANP infusion in normal humans leads to increases in plasma insulin concentration. These authors did not determine if this was a direct or indirect effect of ANP, but it presents the intriguing possibility that insulin and ANP may be involved in a feedback loop.

There have been a few studies that found no difference in plasma ANP levels between diabetic rats (74, 86) or diabetic humans (28, 114) versus control subjects. This disparity in reported ANP levels in DM could be related to differences in the duration of disease, glycemic control, insulin dosage, rat strain, anesthetic, and blood sampling methods (2).

Regarding the finding in this study that atrial tissue irANP levels were significantly diminished in the DM group as compared to the normal group (Figure 13), again there are several possible explanations. In order for the tissue concentrations of ANP to be decreased, there must be either a reduction in synthesis, or an increase in release leading to depletion, or a combination of the two (151). There have been few studies (31, 75, 151) which attempted to quantitate

atrial tissue ANP concentrations in DM, and it has not been determined whether stimulated release or decreased synthesis is the cause of the diminished tissue ANP levels. Hebden et al. (75) and Todd et al. (151) found decreased atrial tissue granularity in STZ-induced diabetic rats with diabetes of 6 and 12 weeks duration, respectively. They used a quantitative stereological technique to determine granularity, as opposed to direct tissue measurement. The electron micrographs from this study seemingly support this finding, but no attempt was made to specifically quantitate the granularity in the atria from DM and normal rats. Chua et al. (31) found decreased atrial tissue levels in rats with alloxan-induced DM of only three days duration. They measured tissue ANP levels with an RIA procedure similar to the method used in this investigation. These observations may be pertinent to some aspects of the decreased ANP levels in diabetic cardiac tissue, but whether or not ANP release is stimulated in DM leading to increased plasma ANP levels is unclear. It is known that protein synthesis is diminished in the cardiac tissue of diabetic rats with diabetes of 7-10 days duration (167). Dillmann (48) reported that diabetes leads to a marked decrease in many cardiac myocyte mRNA's, not all of which have been identified. Thus, it seems reasonable to assume that the synthesis of ANP may also be diminished in DM. However, recently Matsubara et al. (107) reported that ANP mRNA levels are increased in the atria and ventricles of STZ-induced diabetic Wistar-Kyoto rats. Further experimental work in this area would be of considerable interest.

Finally, this study has also identified a substantial subsensitivity of renal response to ANP infusion. The renal response to ANP infusion corresponded to literature reports in the normal group (15, 50, 120), with urine flow increasing approximately 6-fold, and urine sodium and potassium excretion rates increasing approximately 8-fold and 3-fold, respec-

tively. The renal response of the DM group to ANP infusion, however, was markedly blunted. The DM group showed no significant increases in urine flow or urine sodium and potassium excretion rates with ANP infusion. This finding is in agreement with the findings of several reported investigations in both human diabetics (68, 160) and STZ-induced DM in rats (15, 120). The reason for this impaired renal responsiveness to exogenously administered ANP is uncertain, but it may be due to receptor down-regulation in response to elevated plasma ANP levels (15, 120). Circulating ANP levels are known to determine receptor density in target tissues (12), and Benigni et al. (15) demonstrated that the density of renal cortical ANP receptors is decreased in STZ-induced diabetic rats in conjunction with increased plasma ANP levels and decreased response to ANP infusion. Benigni's group found no decrease in ANP receptor affinity in diabetic rats. They hypothesized that the decreased renal response to ANP infusion in DM is due to the decreased number of renal cortical ANP receptors, and that this diminished capability for response may represent a compensatory mechanism to modulate the deleterious effects of hyperfiltration. In other words, lack of response to ANP in this situation may protect the diabetic from a more rapid deterioration of renal function that might occur if the kidney responded normally to ANP, since glomerular hyperfiltration can contribute to glomerular structural damage leading to glomerulosclerosis (15). In contrast, Sahai et al. (134) reported that the lack of response to ANP in DM may contribute to nephropathy. They found that incubation of rat kidney cortex basolateral membranes with ANP led to a dose-dependent increase in Ca^{2+} + Mg^{2+} ATPase activity in normal rats, but ANP had no effect on this parameter in basolateral membranes from STZ-induced diabetic rat kidney cortex. They reasoned that this lack of response in DM may contribute to the development of intra-

cellular calcium overload and nephropathy. In addition, since it has been proposed that elevated plasma ANP levels in diabetes may be contributing to the increased GFR and natriuresis noted in these subjects (117), it seems possible that the kidney may already be functioning at near-maximal capacity. If this is the case, then the kidneys may be unable to respond to further increases in plasma ANP.

In this study there were no differences between the normal and DM groups or within the groups with regard to heart rate and MABP changes in response to ANP infusion. This corresponds to findings in human (68, 160) and animal (120) studies, which have reported impaired renal responses to ANP infusion in diabetic subjects without evidence of diminished cardiovascular response. Haak et al. (68) and Walter-Schrader et al. (160) reported that diabetic and normal subjects responded to a bolus injection of ANP with similar decreases in MABP and no change in heart rate. Interestingly, in these two studies it was also found that ANP infusion produced similar declines in plasma renin activity and plasma aldosterone concentration in both diabetic and nondiabetic subjects, so endocrine response to ANP appears to be unimpaired in human diabetes. Patel and Zhang (120) reported similar cardiovascular results using rats and a sustained infusion of ANP, although the decrease in MABP with ANP infusion was not statistically significant in either the diabetic or normal rats. Patel and Zhang's findings are very similar to the results obtained in this study. The failure of ANP infusion to produce a significant decrease in MABP in these studies is probably a reflection of the low infusion doses used. The animals in these studies were not hypertensive, and this may also have contributed to the lack of significant hypotensive response to ANP infusion, since ANP is reported to have more dramatic hypotensive actions in hypertensive subjects (154).

It is concluded from this investigation that streptozotocin-induced diabetes mellitus of approximately three weeks duration in rats results in increased plasma irANP and decreased atrial tissue irANP levels, as well as diminished renal responsiveness to exogenously administered ANP. Determination of the contribution of elevated plasma ANP levels to abnormalities often associated with DM, such as glomerular hyperfiltration and suppression of the renin-angiotensin-aldosterone system, awaits the development of specific ANP antagonists. In addition, the mechanisms that result in elevated plasma ANP levels and diminished atrial tissue ANP levels remain to be elucidated. Pharmacokinetic studies of ANP distribution and clearance in diabetic versus normal rats would help determine the cause of elevated plasma ANP levels in diabetic rats. Likewise, investigations on the rate of ANP synthesis in atrial tissue in normal and diabetic rats would be beneficial in determining the cause of decreased atrial tissue ANP levels in diabetic rats. It would also be interesting to use a more chronic model to investigate the effects of diabetic cardiomyopathy and cardiac autonomic neuropathy on ANP plasma and tissue levels, as well as target tissue responses. The similarity in body systems affected by both ANP and DM make it seem likely that ANP is involved in some of the alterations of this disease. A definitive relationship between the function of ANP and the pathophysiology of DM awaits further investigation.

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- Sophomore Biology Award
- General Chemistry Award
- Distinguished Scholar Scholarship

PROFESSIONAL ORGANIZATIONS

American Veterinary Medical Association
American Physiological Society

PUBLICATIONS

BLACK, L. S., and J. C. LEE. Atrial natriueretic peptide and streptozotocin-induced diabetes in rats. FASEB J. 5: A671. 1991.

BLACK, L. S., and J. C. LEE. Effects of STZ-induced diabetes on atrial natriuretic peptide levels in rats. In: Progress in Atrial Peptide Research, Vol. 3, edited by B. Brenner and J. Laragh. New York: Raven Press, 1989, pp 396-399.

BLACK, L. S., and J. C. LEE. Effects of STZ-induced diabetes on atrial natriuretic peptide levels in rats. Am. J. Hypertension 1988: 1: 112A. (Abstract).