Natriuretic Peptides As A Humoral Link Between The Heart And The Gastrointestinal System

by

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DEDICATION

I dedicate this thesis to my loving family, my wife Sirgut Tirusew and my children Leeyu and Yonaas, my parents Addisu Merdassa and my mom Tsedale Sahlemariam. Your, dedication, sacrifices, prayers and love has made this possible and for that I am eternally thankful.
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TABLE OF CONTENTS

LIST OF TABLES iv
LIST OF FIGURES v
ABSTRACT viii

CHAPTER ONE – INTRODUCTION AND BACKGROUND

   Body Fluid Volume Sensing and Regulation 1
   The Natriuretic Peptides 5
   Natriuretic Peptide Receptors 11
   Tissue Distribution of Natriuretic Peptides and their Receptors 15
   Physiological Effects of the Natriuretic Peptides 15
   Gastrointestinal Effects of Natriuretic Peptides 17

CHAPTER TWO – MATERIALS AND METHODS

   Measurement of Gastric Contractility and Intragastric pressure 22
   Experimental Animals 22
   Surgical Preparation 23
   Positive and Negative Controls 25
   Blood Pressure Measurement 28
   Measurement of Gastric Emptying 28
   Measurement of Absorption 29
   Induction of Myocardial Ischemia (Myocardial Injury) 31
LIST OF TABLES

Table 1  Composition of modified Krebs’s solution  26
Table 2  Molar concentration of modified Krebs solution  27
Table 3  Peptides used in this study  30
LIST OF FIGURES

Figure 1 Linear peptide structure of the natriuretic peptides 6
Figure 2 Schematic representation of the synthesis of natriuretic peptides ANP, BNP and CNP 8
Figure 3 Schematic representations of the natriuretic peptide receptors 12
Figure 4 A model for natriuretic peptide receptor activation 13
Figure 5 Typical pressure wave pattern of a basal gastric contraction 39
Figure 6 Simultaneous recording of blood pressure and intragastric pressure 40
Figure 7 The effect of intravenous administration of Gherelin on intragastric pressure 41
Figure 8 Three individual experiments showing intragastric pressure after a 10 ng/g iv bolus of ANP, BNP and CNP 42
Figure 9 Typical recoding of post peptide injection gastric contraction wave pattern 43
Figure 10 The effect of C-ANP$_{4-23}$ on gastric pressure 44
Figure 11 BNP did not change gastric pressure in NPR-A knock out mice 45
Figure 12  Determination of baseline and peak intragastric pressure before and after peptide injection 46

Figure 13  Mean reduction in intragastric pressure following intravenous BNP injection 48

Figure 14  Mean reduction in intragastric pressure following intravenous ANP injection 49

Figure 15  Mean reduction in intragastric pressure following intravenous CNP injection 50

Figure 16  The Effect of BNP on gastric emptying 51

Figure 17  Dose dependent reduction of gastric emptying 52

Figure 18  The Effect of BNP on absorption 53

Figure 19  Comparison of plasma fluorescence following intravenous FITC-dextran injection; BNP vs. Vehicle 54

Figure 20  Representative histological appearance of the myocardium 14 days after cryoinfarction 66

Figure 21  Comparison of percent gastric emptying in Sham vs. MI wild Type mice, 1 week post MI 67

Figure 22  Comparison of percent gastric emptying in Sham vs. MI Wild Type mice 68

Figure 23  Comparison of percent gastric emptying in Sham vs. MI NPR-A knockout mice 69

Figure 24  Comparison of absorption measured in relative plasma fluorescence units. Sham vs. MI, WT mice 70

Figure 25  Comparison of absorption measured in relative plasma fluorescence units. Sham vs. MI, NPR-A KO 71
| Figure 26 | Schematic diagram of non muscle myosin-II | 78 |
| Figure 27 | Schematic depiction and electron micrograph of the structures of intestinal villi | 79 |
| Figure 28 | Cytoskeletal structures in the intestinal microvillus | 80 |
| Figure 29 | Ultra structures of the microvilli cytoskeleton | 81 |
| Figure 30 | Transmission Electron micrographs of intestinal villi in various state of contraction | 82 |
| Figure 31 | Schematic depiction of tight junctions | 83 |
| Figure 32 | Toluidine blue stained 3µm sections of a mouse intestinal tissues | 85 |
| Figure 33 | Higher magnification view of jejunal villi | 86 |
| Figure 34 | Electron micrographs of the intestinal microvilli Control vs. BNP treated | 87 |
| Figure 35 | Jejunal villi immunostained for non muscle myosin type IIB – Control | 88 |
| Figure 36 | Comparative images of control vs. BNP treated jejunal villi | 89 |
| Figure 37 | Jejunal villi and smooth muscle bundles immunostained for non muscle myosin | 90 |
| Figure 38 | A 3 µm sections of a mouse jejunum immunostained for non muscle myosin type IIB. Control vs. BNP | 91 |
NATRIURETIC PEPTIDES AS A HUMORAL LINK BETWEEN THE HEART AND THE GASTROINTESTINAL SYSTEM

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ABSTRACT

Natriuretic peptides are a family of hormones released by several different tissues and exert various physiological functions by coupling with cell surface receptors and increasing intracellular cyclic guanylyl monophosphate (cGMP). Atrial Natriuretic Peptide (ANP) and B-type Natriuretic Peptide (BNP) are released in response to mechanical stretch of the atrial or ventricular myocardium, respectively and their plasma level is markedly elevated during myocardial infarction and heart failure. Heart failure in turn is associated with symptoms suggestive of perturbed gastrointestinal function such as nausea, indigestion and malabsorption.

Intragastric pressure was monitored using a balloon catheter in anesthetized mice. The pressure before and after treatment with a 10 ng/g intravenous dose of ANP, BNP, CNP or vehicle was compared and analyzed. All the natriuretic peptides significantly decreased intragastric pressure compared to vehicle. These effects were
attenuated or absent in natriuretic peptide receptor type-A (NPR-A) knockout mice. Furthermore, the effect of BNP on gastric emptying and intestinal absorption was examined using a meal consisting of fluorescence labeled dextran gavage fed to awake mice. BNP significantly decreased gastric emptying and absorption as compared to vehicle control. Using a cryoinfarction acute myocardial injury model, our investigation showed that mice with acute cryoinfarction had a significantly lower gastric emptying and absorption of a gavage fed meal compared to sham. Circulating BNP levels were significantly higher in the infarcted mice compared to controls. Immunostaining showed amplified distribution of the non-muscle myosin type-II (MCH-II) in BNP treated mice. MCH-II is involved in movement of intestinal villi.

In summary, natriuretic peptides in general and BNP in particular, have gastrointestinal effects including reduced gastric contractility, emptying and absorption. In addition to their effect on smooth muscle relaxation mediated by cGMP, natriuretic peptides appear to have an effect on distribution of MHC-II in cells of the intestinal villi.

We postulate that these effects are aimed at mediating a ‘communication’ between the cardiovascular and gastrointestinal systems. Further characterization of such a link will not only add a
dimension to the understanding of the pathophysiology of heart failure
but also enhances the search for further therapeutic targets.
CHAPTER ONE
INTRODUCTION AND BACKGROUND

Since maintaining body fluid volume is one of the most tightly regulated physiological functions, animals have evolved with increasingly sophisticated mechanisms for rapidly detecting and responding to changes in fluid volume. In vertebrates, the vascular low pressure volume sensors are embedded within the walls of the myocardium and large pulmonary blood vessels. These volume sensors detect changes in pressure (or volume) induced stretch of the vessel and myocardial wall. Signals from these receptors travel via afferent fibers of the vagus nerve to the solitary tract nucleus of the medulla oblongata in the brain stem (Donald & Shepherd, 1979; Thorén et al., 1976). Feedback activation or deactivation of neural signals back to the target organs then modulate mechanisms that result in more or less diuresis, natriuresis or vascular tone depending on the body's requirement. However even as early as the first half of the last century there were studies that suggested there may be humoral (non neuronal) system of receptors that responded to the change in
pressure or volume stretching the atrial myocardium. For instance, expansion of plasma volume by blood transfusion to healthy dogs was shown to increase urine flow (Metcalf, 1944). Similarly infusion of isoncotic solution of albumin was shown to produce marked diuresis in human volunteers (Welt & Orloff, 1951). Strauss et al reported that infusion of isotonic (0.9%) saline to healthy volunteers increased “water diuresis” during recumbency; and they noted that the body position influenced how the infused volume is distributed and sensed by the atrium (Strauss et al., 1951). The role of atrial stretch in such volume induced diuresis was already experimentally established in the mid 1950s (Henry & Pearce, 1956). Henry and colleagues used a balloon to distend the atrium in dogs and showed that urine output was increased corresponding to the degree of atrial distension. In their paper entitled “The possible role of cardiac atrial stretch receptors in the induction of changes in urine flow”, Henry and Pearce noted that vagotomy did not abolish the diuretic response to isotonic infusions; and this led to their conclusion in 1956 that “the body may be provided with other receptors or pathways by which it receives information which makes possible the regulation of blood volume by the control of urine flow”.

Nevertheless, until only a little over two decades ago; the outcome of the activation of atrial and venous stretch receptors was
believed to almost entirely depend on the modulation of autonomic nerve activity or varying levels of secretion of antidiuretic hormone (ADH) from the neurohypophysis.

In 1980 Adolfo de Bold reported his seminal discovery that intravenous injection of extract from the rat atrium produced a marked diuresis (de Bold et al., 1981).

Thus it became apparent that the atrium produced a diuretic and natriuretic substance that was released into the circulation and worked through a receptor system that is distinct from the neurally mediated feedback inhibition. This natriuretic ‘substance’ was initially termed atrial natriuretic factor (ANF). With this discovery, it also became apparent that the heart has an endocrine function and directly ‘communicates’ with the kidney by virtue of its own cardiac hormone systems.

The presence of granular structures in the atrial myocytes of several different species was one of the earliest findings of the advent of electron microscopy (Jamieson & Palade, 1964; Kisch, 1956; Palade, 1961). Moreover, the possible relationship between atrial granules and the degree of sensitivity of the atrium to volume or salt loading was suggested as early as 1976 (Marie et al., 1976). However it was with de Bold’s seminal experiments that the content of these atrial granules was clearly hinted to be a potent natriuretic substance that directly
influenced renal handling of salt and water (de Bold et al., 1981; de Bold., 1985). The atrial natriuretic factor was later determined to be a peptide produced by the atrial myocardium (Currie et al., 1984; Flynn TG, 1983; Misono et al., 1984) and it is currently more commonly referred to as Atrial Natriuretic Peptide (ANP). The ANP gene was subsequently sequenced revealing the remarkable similarity and genetic conservation of the natriuretic peptides across many species (Seidman et al., 1984).
The Natriuretic Peptides

The identification of ANP in 1985 was soon followed by the discovery of a similar peptide in porcine brain (Sudoh et al., 1988) hence termed as brain natriuretic peptide (BNP). However it was later discovered that BNP was another cardiac hormone and is actually absent in brains of some species (Ogawa et al., 1991; Ogawa et al., 1990). In 1990, C-type natriuretic peptide (CNP) was isolated from the brain of pigs, bullfrogs and two species of teleost fishes (Sudoh et al., 1990; Suzuki et al., 1991; Yoshihara et al., 1990). The three natriuretic peptides share a common structural feature, a conserved 17 amino acid ring with variable N- and C-terminal sequences (figure 1). The number of amino acid residues extending from the C terminal is usually 5 for ANP, 6 for BNP and 0 for CNP with few exceptions. Thus the C-terminal sequence appears to be a major determinant of biological activity of the natriuretic peptides. CNP is the most conserved of the three peptides across species and it is believed to have evolved earlier in the phylogenetic tree, ANP and BNP being derived from it at a later point in evolution. In recent years a fourth natriuretic peptide, a 38 amino acid peptide known as deandropsis natriuretic peptide (DNP) has been isolated from the venom of the green mamba snake (Munagala et al., 2004). Immunoreactivity to DNP has been shown in human and rodent plasma (Johns et al., 2007;
Schirger et al., 1999). However, a gene coding for it has not yet been isolated and the physiological significance of DNP in humans (if any) remains poorly understood.

**Figure 1. Linear peptide structure of the natriuretic peptides**

![Linear peptide structure of the natriuretic peptides](image)

Adapted from (Cea, 2005)

In humans, the ANP and BNP genes are localized in tandem on chromosome 1. Transcription of the ANP gene yields an mRNA that encodes a 151 amino acid metabolic precursor known as preproANP.
PreproANP is rapidly converted to a 126 amino acid peptide proANP. ProANP is the predominant storage form of ANP and the major constituent of atrial granules. Atrial distension is the major signal for the release of ANP (Anderson et al., 1986; Dietz, 1984; Dietz et al., 1991; Kinnunen et al., 1992; Sato et al., 1986). When such a signal is sensed; proANP is cleaved by a cardiac serine protease (corin) into an amino fragment pro-ANP (amino acids 1-98) and ANP, the biologically active fragment (amino acids 99-126) (Bloch et al., 1986; Vuolteenaho et al., 1985). Further cleavage of proANP (1-98) results in more peptide fragments (1-30) and (31-67); these fragments also have biological activity and neutralization of proANP 1-30 was shown to exacerbate hypertension in spontaneously hypertensive rats (Dietz et al., 2001; Dietz et al., 2003; Dietz et al., 1995; Dietz & Villarreal, 1995; Vesely et al., 1999). The amino acid sequences of the main biologically active hormone ANP 99-126 are identical in all mammalian species except at residue 110, which is methionine in humans (Kangawa et al., 1984; Lewicki et al., 1986; Vlasuk et al., 1986) but isoleucine in rats, mice and rabbits (Oikawa et al., 1985; Seidman et al., 1984; Yamanaka et al., 1984). Alternate processing of the prepro ANP in the kidney produces a 32 amino acid peptide known as urodilatin. Urodilatin is secreted into the lumen of the distal nephron (medullary collecting duct) where it is believed to be involved in
regulation of sodium and water absorption in the kidney (Forssmann et al., 1998; Kuhn, 2005). Physiologically plasma ANP is markedly increased in response to pressure or volume overload or in pathological states such as heart failure or ventricular hypertrophy. However the plasma half life is rather short, averaging less than 3 minutes which indicates that the release of ANP serves to counteract the effect of acute pressure or volume overload (Lang et al., 1985; Ruskoaho, 1992).

**Figure 2. Synthesis of natriuretic peptides and prohormones**

![Diagram of natriuretic peptides and prohormones]

Adapted from Koller and Goeddel 1992
Human BNP is produced as a 132 amino acid residue preproBNP that is subsequently cleaved to a 108 amino acid prohormone. Additional cleavage yields the 32 amino acid active hormone and an inactive 76 amino acid amino terminal (NT) fragment sometimes known as NT-proBNP (Saito et al., 1989; Seilhamer et al., 1989; Sudoh et al., 1988). Ventricular BNP is not stored in granules in the myocytes, instead BNP production is regulated at the transcription level by various stimuli, the main stimulus for BNP synthesis is stretch of the ventricular wall by volume and/or pressure overload (Grépin et al., 1994; Thuerauf et al., 1994). Even though the BNP gene is located in tandem with the ANP gene; BNP expression doesn’t always parallel ANP gene expression. The response of the BNP gene is quicker than that of ANP suggesting a more acute and sustained role for BNP in response to a volume overload. Increase in BNP mRNA is detected within one hour of increased ventricular wall tension induced by increased venous volume or acute myocardial infarction (Hama et al., 1995; Nakagawa et al., 1995). BNP has a much longer half-life (than ANP) of about 20 minutes (Espiner et al., 1995) and with sustained cardiac stress; as in the case of heart failure, BNP mRNA levels have been shown to remain increased (Tokola et al., 2001). BNP secretion takes two forms, constitutive, where the BNP is secreted as fast as it is being formed and a regulated pathway where the BNP is stored in
granules prior to being secreted (Kelly, 1985). Ventricular myocytes shift between the two pathways depending on the stimulus, the constitutive secretion being called upon when there is acute need for BNP secretion and the regulated pathways operating when there is a sustained myocardial stress such as chronic heart failure (Bloch et al., 1986; Kelly, 1985).

CNP is mainly expressed in the brain, chondrocytes and endothelial tissue. Myocardial tissue has a much smaller amount of CNP than ANP or BNP and CNP is not stored in granules (Yandle, 1994). In humans, proCNP contains 103 amino acid residues and it is cleaved to a 53 amino acid CNP in the brain, the heart and endothelial tissue. Further cleavage yields a 22 amino acid peptide which is the predominantly circulating form of CNP (Stingo et al., 1992; Totsune et al., 1994; Wu et al., 2003). While CNP 53 is believed to be predominantly a neurotransmitter, it is also involved in bone and cartilage growth; CNP 22 is mainly involved in autocrine and paracrine regulation of vascular tone (D'Souza et al., 2004). CNP has less effect on diuresis and natriuresis than ANP or BNP and a more potent effect on smooth muscle relaxation, it is thus believed to be mainly involved in the regulation of coronary vascular tone in the heart (Clavell et al., 1993; Komatsu et al., 1992; Sudoh et al., 1990).
**Natriuretic Peptide Receptors**

The natriuretic peptide receptors are members of the transmembrane guanylyl cyclase family of enzymes that are widely distributed in human and animal tissues. There are at least seven different guanylyl cyclase enzymes identified so far (Anand-Srivastava & Trachte, 1993; Garbers et al., 2006). Natriuretic peptide receptor (NPR) types A and B (NPR-A and NPR-B) are structurally similar and exist as homodimers or homotetramers in intact mammalian cells (Chinkers & Wilson, 1992; Iwata et al., 1991; Katafuchi et al., 1994). The receptor consists of an extra cellular ligand-binding domain, a membrane spanning domain and intracellular kinase-like and guanylyl cyclase domains (Nagase et al., 1997; Potter, 2005). Both ANP and BNP bind to NPR-A although ANP is known to bind with at least 10 times more affinity to NPR-A (Kambayashi et al., 1990; Nakao et al., 1991). NPR-B has similar structure to NPR-A, but selectively binds to CNP. Natriuretic peptide receptor type-C (NPR-C) has a short intracellular sequence that doesn’t have a guanylyl cyclase domain.
Figure 3. Schematic representation of the natriuretic peptide receptors

Adapted from Potter et al., 2006

Ligand binding to the receptor leads to the activation of the guanylyl cyclase and results in a conformational change. The C-terminal guanylyl cyclase then comes into a tight association that leads to conversion of guanosine triphosphate (GTP) to 3’5’- cyclic guanosine monophosphate (cGMP) (Chinkers & Wilson, 1992; Foster et al., 1999). The ATP binding site in the kinase homology domain (KHD) is believed to be essential for ligand-induced signal transduction and
deletion of the KHD depresses the guanylyl cyclase catalytic region (Chinkers & Garbers, 1989). Phosphorylation of amino acid residues in the KHD of the receptor is essential for normal function and dephosphorylation appears to be one method of the natriuretic peptide receptor desensitization (Potter & Garbers, 1992; Potter & Hunter, 1998).

Figure. 4. A model for natriuretic peptide receptor activation

Adapted from Silberbach and Roberts 2001
The end result of these mechanisms of receptor activation is increased concentration of intracellular cGMP; cGMP in turn exerts its physiological effects by binding to one of three cGMP binding proteins. cGMP dependent protein kinases (PKG), cGMP binding phosphodiesterases (PDE) and cyclic nucleotide gated ion channels (Pfeifer et al., 1996; Rybalkin et al., 2003; Smolenski et al., 1998).

As mentioned earlier, natriuretic peptide receptor type-C (NPR-C) has a short intracellular sequence that doesn’t have a guanylyl cyclase domain. The major role of NPR-C was initially believed to be as a clearance receptor and regulation of the plasma concentration of ANP and BNP through receptor mediated internalization and degradation (Matsukawa et al., 1999). However recent evidence suggests that NPR-C is involved in signaling that leads to reduction of adenylyl cyclase activity through activation of inhibitory G protein (Gi) (Anand-Srivastava & Trachte, 1993; Rose & Giles, 2007).

The natriuretic peptides are cleared from the circulation by three mechanisms. Endocystosis and degradation by coupling with NPR-C, enzymatic cleavage by neutral endopetidases and by glomerular filtration and excretion in the urine (Boerrigter & Burnett, 2004; Freda & Francis, 2006).
**Tissue Distribution of Natriuretic Peptides and their Receptors**

The atrial and ventricular cardiomyocytes are the main sites of production and storage of ANP and BNP, respectively. This was confirmed through seminal experiments that showed that atrial appendectomy resulted in marked reduction of both the plasma levels of ANP and the diuretic and natriuretic effect seen in response to a volume load with isotonic saline (Veress & Sonnenberg, 1984; Villarreal et al., 1986). Nevertheless, ANP and BNP mRNA have been detected in several non-cardiac tissues including the adrenal glands, the kidneys, lung, the gonads, lymphoid tissue and the gut (Gerbes et al., 1994; Gower et al., 1994; Gower et al., 2003; Li et al., 2006; Nguyen et al., 1990; Sharkey et al., 1991; Vollmar, 1990). Natriuretic peptide receptors have also been detected in the gastrointestinal (GI) tract of both non-mammalian and mammalian species including humans (Gower & Skvorak, 1997; Li & Goy, 1993; Lowe et al., 1989; Ohyama et al., 1992; Schulz et al., 1998).

**Physiological Effects of the Natriuretic Peptides**

Vascular relaxation is one of the most important physiological actions of the natriuretic peptides. Binding of cGMP to PKG is known to regulate ion channels in vascular smooth muscle cells with a cascade of molecular events that culminate in a lower concentration of
intracellular calcium. These effects include reduction of calcium influx, increase of calcium efflux and promotion of calcium sequestration in sarcoplasmic reticulum (Tamaoki et al., 1997). Reduced vascular tone and vasodilatation in turn results in lower total peripheral vascular resistance which has important physiological benefit on the cardiovascular system; especially in the face of cardiac ischemia or volume overload. Such vascular relaxation and vasodilatation will also result in reduced pressure in the renal afferent arterioles thereby increasing glomerular filtration, promoting diuresis and natriuresis. In addition to these effects on vascular smooth muscle cells, the direct effect of ANP and BNP on renal tubules and mesangial cells as well as inhibition of the renin angiotensin aldosterone (RAAS) system results in decreased sodium absorption from the renal tubules (Lohmeier et al., 1995; Zeidel, 1993).

ANP and BNP are also known to decrease sympathetic outflow and catecholamine release from peripheral sympathetic neurons which benefits the cardiovascular system since it leads to lower blood pressure, decreased heart rate and natriuresis (Levin et al., 1988). Further support for the importance of the natriuretic peptides in blood pressure and blood volume regulation comes from data showing that NPR-A transgenic mice show chronic hypertension and ventricular hypertrophy (John et al., 1995). NPR-A transgenic mice were recently
shown to have an upregulation of the angiotensin converting enzyme (ACE) and angiotensin II type 1a receptor (AT1) mRNA by up to four-fold signifying the importance of the natriuretic peptides in counteracting the effect of the renin angiotensin aldosterone (RAS) system (Vellaichamy et al., 2007). It is also shown that NPR-A knockout mice exhibit dysregulation of matrix metalloproteinases and tissue inhibitors of metalloproteinases; enzymes involved in the regulation of collagen synthesis and organization of myocardial fibrils (Li et al., 2000; Spinale, 2002). As a result NPR-A knockout mice show increased tendency towards myocardial fibrosis, hypertrophy and eventually heart failure that leads to premature death as compared to the wild type mice.

**Gastrointestinal Effects of Natriuretic Peptides**

Natriuretic peptides are known to have several effects on contractile and absorptive functions in the GI tract. ANP has been shown to inhibit intestinal sodium and water absorption in teleost and mammalian intestines (Barros et al., 1990; Matsushita et al., 1991; O'Grady et al., 1985). A decrease in jejunal water absorption in response to intravascular volume overload was also shown in rats. Such a decrease in transjejunal water absorption was absent in rats that underwent right atrial appendectomy and this effect returned
when ANP was exogenously administered (Pettersson & Johnsson, 1989). Intravenous administration of BNP and CNP has also been shown to decrease jejunal electrolyte and water absorption in dogs (Morita et al., 1992). Earlier investigations have also shown that injection of either ANP or BNP into the cerebral ventricles inhibits thirst induced by water deprivation or angiotensin (Antunes-Rodrigues et al., 1985; Itoh et al., 1988; Zhu & Herbert, 1996). Furthermore, ANP and BNP have been shown to have an indirect effect on uptake and excretion of sodium and water through inhibition of vasopressin and aldosterone secretion (Januszewicz et al., 1986; Nguyen et al., 1989).

The effect of natriuretic peptides on contractility of GI smooth muscle has been documented beginning with the early discovery of these peptides and their receptors in the GI tract (Scott & Maric, 1991). Subsequent studies have confirmed these findings showing that ANP, BNP and CNP all inhibit contractility of isolated gastric and intestinal smooth muscle cells from different species including humans (GuoCui et al., 2003; GuoJin et al., 2003; Yasuda et al., 2000).

Apart from the traditional ‘cardiac’ natriuretic peptides ANP, BNP, and CNP; the GI tract is also known to be a source of two peptides with structures very similar to the natriuretic peptides. Guanylin and
uroguanylin were first isolated from rat intestine and opossum urine
and later found to be widely distributed in non-mammalian and
mammalian species including humans (Beltowski, 2001; Date et al.,
1998). In humans, uroguanylin is mainly expressed in the
enterochromaffin (EC) cells of the duodenum (where ANP is also
expressed), whereas guanylin is expressed in the jejunum and the
colon (Beltowski, 2001; Li et al., 2006). Uroguanylin is secreted in
response to oral salt load and the circulating hormone is known to
mediate sodium balance in the post-prandial state by increasing renal
excretion of sodium and potassium while the luminally secreted
hormone leads to increased chloride and bicarbonate secretion in to
the lumen of the intestine (Beltowski, 2001; Forte et al., 1996).
Uroguanylin and guanylin mRNA are detectable in the atrial and
ventricular myocardium and interestingly, plasma levels of guanylin
and uroguanylin are increased during heart failure and renal failure
(Beltowski, 2001; Forte et al., 1996). Both guanylin and uroguanylin
receptors are membrane bound guanylyl cyclase enzymes and
activation of these receptors leads to increased intracellular cGMP
(Carrithers et al., 1999; Forte et al., 1996; Forte et al., 1999). Both
synergistic and antagonistic interaction between ANP and BNP on one
hand and uroguanylin and guanylin on the other have been shown and
there has been some suggestion that this evidence points to a
probable regulatory link between the kidney and the GI tract in the
process of sodium and water balance (Santos-Neto et al., 2006).

Taken together, the remarkable genetic conservation of the
natriuretic peptides, the stimuli for their release and their effect on
sodium and water uptake is strong evidence that they serve crucial
physiological functions that conferred survival benefit earlier in
evolutionary times and have since evolved to be important conveyers
of signals among the various organ systems involved in cardiovascular
homeostasis.

While the cardio-renal link is now sufficiently well established,
there have been very few studies conducted to assess the functional
significance of the effect of natriuretic peptides on the GI tract and
how this all fits in the bigger scheme of volume regulation. The early
observations on the effect of natriuretic peptides as well as recent
evidence showing the presence and role of a possible ‘intestinal’
natriuretic peptide system raise several intriguing questions. If the
heart sends humoral signals to the kidney to decrease sodium
absorption, shouldn’t it also use the same signals to limit sodium and
water absorption from the GI tract? GI symptoms such as nausea,
indigestion and malabsorption are frequent clinical findings in the face
of acute heart attack or chronic heart failure, states where BNP levels
are elevated. Could BNP be responsible for some of these symptoms?
If so, could there be potential targets of therapy for heart failure in the GI tract?

Our studies will provide evidence that the effect of natriuretic peptides on gastric and intestinal smooth muscle cell contractility has a functional dimension. By directly testing the effect of ANP, BNP and CNP on gastric contractility, gastric emptying and absorption we show that these peptides do decrease gastric emptying and absorption. Moreover BNP, a peptide with increasing clinical utility, is shown here to cause decreased gastric emptying and intestinal absorption following acute myocardial injury using a whole animal cryo induced acute MI model. While our data suggests that these effects may be mediated by NPR-A, we also show data that suggests a more direct effect on absorptive structures at the level of intestinal microvilli. Further characterization of the signals involved in these functions not only adds a new dimension to cardiovascular research but could also lead to identification of new therapeutic targets for the treatment of heart failure.
CHAPTER TWO
MATERIALS AND METHODS

Measurement of Gastric Contractility and Intragastric Pressure

These protocols were approved by the University of South Florida Institutional Animal Care and Use Committee.

Experimental Animals

NPR-A Knockout (KO) mice were obtained from our resident colony that was founded with pathogen-free breeding pairs and were genetically monitored by PCR of tail-snip DNA. The generation of NPR-A knockout mice has previously been described in detail (Lopez et al., 1995). Wild type (C57BL/6) mice were purchased from commercial sources. The wild type (WT) mice were all males with ages ranging from 8 to 12 weeks and weight ranging from 18-26 grams at the time of the experiment. The NPR-A KO mice ranged from 10 to 32 weeks in age and 24-38 grams in weight at the time of the experiment, with equal number of male and female KO mice in the experiment and vehicle group. The parental strain of the knockout mice was C57BL/6.
**Surgical Preparation**

In order to objectively determine the effect of natriuretic peptides on gastric contractility, a method of measuring intragastric pressure before and after peptide injection was developed as follows. The mice were anesthetized with sodium pentobarbital (0.9 mg/10 gm body weight) given intraperitoneally (ip) and supplemental doses of 0.5 mg given ip as needed. The mice were then placed on a temperature controlled surgical table and a tracheotomy was performed using a 20 ga. Luer-stub adapter. The right jugular vein was catheterized with polyethylene (PE)10 tubing for injections and infusions and the right carotid was catheterized with a 5 cm piece of PE tubing (o.d. 0.012”, i.d. 0.006”: Braintree Scientific, Inc.) attached to a 12-18” segment of PE 50 tubing for arterial blood pressure measurements. A 10 mm left sub-costal skin incision was made to access the stomach. A 3 mm vertical incision of the stomach fundus was made with cautery carefully choosing a site that has minimal or no visible blood vessels. A 2-3 mm latex balloon fitted with PE tubing and primed with saline was inserted into the stomach. The balloon was then minimally distended by adding 20-25 µl of saline and attached to a standard pressure transducer (Gould/Statham DB25). The intragastric catheter was held in place by the minimal distension of the balloon and suturing the stomach incision was not necessary. The skin
incision was closed with 1-2 interrupted surgical sutures. Following these surgical preparations, the mice received a 100 µl bolus of 0.9% saline intravenously (iv) via the jugular catheter and then allowed a one hour equilibration period while being infused with 0.9 % saline iv at 5 µl/minute. The saline bolus and infusion were administered to compensate for blood loss associated with the surgical procedure and maintenance fluid requirement. Arterial blood pressure, heart rate and intragastric pressure were monitored continuously via the carotid and intragastric catheters and recorded on a data acquisition system (DATAQ Instruments, Akron, OH).

The change in intragastric pressure was measured as the difference between the peak and baseline pressure. The measurements were taken for three 30 minute periods. The basal gastric pressure was measured from 30 to 0 minutes before injection. Post injection period was from 10 to 40 minutes after injection to correspond with the peak plasma level of the peptides and the recovery period was from 90 to 120 minutes after injection corresponding to the period later than 5 peptide half-lives (12). The changes in intragastric pressure during each of the three periods were averaged for each mouse and the differences in intragastric pressure between the experimental and vehicle groups during the three periods were compared using a one way ANOVA with Fisher’s least significant
difference test (LSD) used as a post hoc test. A “p” value of <0.05 was considered the criteria for statistical significance.

**Positive and Negative Controls**

Gherelin, a hormone with known prokinetic gastrointestinal effect in rodents, was used as a positive control to ascertain that the waves of contraction we observed and recorded were indeed changes in gastric motility (Depoortere et al., 2005). Gherelin (Rat, Phoenix pharmaceuticals, C# 031-31, Lot # 423341) was administered at a dose of 50 µg/kg body weight in 100 µl of vehicle iv, a dose previously established to increase gastric motility in rodents.

The vehicle was used as a negative control. The vehicle consisted of modified Krebs-Hensledt bicarbonate buffer equilibrated final PH = 7.4, dissolved in the order shown in table 1.
Table 1. Composition of modified Kreb’s solution (g/l)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂</td>
<td>0.220g</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.144g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.224g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.163g</td>
</tr>
<tr>
<td>NaCl</td>
<td>6.72g</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>2.1g</td>
</tr>
<tr>
<td>Glucose</td>
<td>1g</td>
</tr>
<tr>
<td>Albumin</td>
<td>1g</td>
</tr>
</tbody>
</table>
Table 2. Millimolar concentration of modified Kreb’s solution

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Na+</td>
<td>140</td>
</tr>
<tr>
<td>K+</td>
<td>4.2</td>
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<tr>
<td>Ca$^{2+}$</td>
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<td>25</td>
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<tr>
<td>H$_2$PO$_4^-$</td>
<td>1.2</td>
</tr>
<tr>
<td>SO$_4$</td>
<td>1.2</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.5</td>
</tr>
<tr>
<td>Albumin (BSA fraction V)</td>
<td>0.1%</td>
</tr>
</tbody>
</table>
**Blood Pressure Measurement**

Mean arterial pressure was continuously monitored using an intra-carotid catheter and recorded. Average blood pressure was measured for the same three periods as for the gastric pressure measurement. Pre vs. post peptide injection blood pressures were compared to ascertain that our findings were not confounded by differences in blood pressure.

**Measurement of Gastric Emptying**

Conscious WT and NPR-A KO mice (n = 5 in each group) were given BNP at dose of 10 ng/g through the tail vein dissolved in 100 µl of vehicle (modified Krebs solution) or the vehicle alone and immediately gavaged with 0.1 ml of 0.5 Mmol 70 kDa fluorescein-isothiocyanate (FITC)-dextran. The 70 kDa FITC-dextran is known to be non-diffusible across intestinal membrane thus suitable for measurement of emptying (Thorball, 1981). Thirty minutes after the gavage meal, the animals were euthanized and the stomach was separated and the intestine divided into 8 equal segments, each flushed with 3 ml of PBS and centrifuged for 10 minutes. Fluorescence of the supernatant fluid was measured and the percent gastric emptying rate was compared in BNP treated vs. control for both WT and NPR-A KO mice. This method of evaluating gastric emptying has been previously established (Aube et al., 2006).
**Measurement of Absorption**

Conscious WT & NPR-A KO mice (n = 5 in each group) were given 10 ng/g of BNP through the tail vein dissolved in 100 µl of vehicle (modified Krebs solution) or the vehicle alone. The mice were then gavaged with 0.01 ml/g of a solution containing 22 mg/ml of 4 kDa FITC-dextran. Blood was collected via cardiac puncture under pentobarbital anesthesia. Fluorescence was quantified using relative fluorescence units in the plasma. The plasma fluorescence measured 1 hour after gavage feeding in WT and NPR-A KO mice was compared for BNP treated vs. control. Similar comparisons were made for the subsequent experiments between sham vs. Mi in both WT and NPR-A KO mice. The group differences were analyzed using a t-test with p<0.05 considered the significant level for statistical difference. This method of evaluating gastric absorption has been previously established (Aube et al., 2006).

We also compared the fluorescence of a 50 µl plasma sample taken 1 hour after iv administration of 100 µl of 0.5 mmol 4kDa FITC-dextran in BNP treated vs. vehicle WT mice. The purpose of this test was to rule out the possibility that the changes in plasma fluorescence were produced by other actions of BNP such as increased excretion, redistribution or metabolism of the dextran as this effect of BNP has been previously established (Huxley et al., 1987).
The concentration of fluorescein was determined using a fluorimeter (FLUOstar Galaxy, BMG Labtechnologies) with an excitation wavelength at 485 nm and an emission wavelength of 520 nm using serially diluted samples of the marker as standard.

**Table 3. Peptides used in this study**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANP</td>
<td>Rat ANP, Sigma, P # A8208</td>
</tr>
<tr>
<td>BNP</td>
<td>Rat BNP-32, Phoenix Pharmaceuticals, 011-14, Lot # 421752</td>
</tr>
<tr>
<td>CNP</td>
<td>Rat 32-53, Bachem, P # H-1296, Lot # B00656</td>
</tr>
<tr>
<td>c-ANF 4-23</td>
<td>Rat, Phoenix Pharmaceuticals</td>
</tr>
<tr>
<td>Gherelin</td>
<td>Rat, Phoenix Pharmaceuticals, C # 031-31, Lot # 423341</td>
</tr>
</tbody>
</table>

In the initial experiments, the peptides were administered intravenously at 10 ng/g body weight. This dose was chosen to raise and sustain the plasma levels above 500 pg/ml; a level that is consistent with a greater than 90% likelihood of heart failure (for BNP) in humans (Maisel & Mehra, 2005). For subsequent dose response experiments we administered BNP at 1, 5, 10 and 100 ng/g body weight iv.
**Induction of Myocardial Ischemia (Myocardial Injury)**

Wild-type and NPR-A knockout mice were anesthetized with 2-3% isoflurane-oxygen flow at 500ml/minute. A 2 mm incision was made through the skin, just distal to the 4th and 5th intercostal space. Blunt dissection through all thoracic musculature, using a fine-tipped instrument was performed to accommodate passage of a probe induction catheter (PIC). Using digital pressure, the distal tip of the PIC (catheter cap in place) was flattened as much as possible (to aid in atraumatic insertion), and the PIC was then passed through the muscle wall. Prior to removing the cap, the operator pinched the proximal tip of the PIC shut (to aid in the prevention of pneumothorax). The oxygen flow was then turned up to 2 L/min. The liquid-nitrogen-cooled probe was then quickly threaded through the PIC, to the full length of the PIC. If needed, the PIC was then gently repositioned so the bevel and the cooled probe were in direct contact with the left ventricle of the heart. Correct placement of the probe, with subsequent cardiac thermal injury, was confirmed as the super cooled probe "grabbed" the warmer tissue of the heart, and remained affixed until the probe had warmed enough for release. After passive release, the probe and PIC, as one unit, were quickly withdrawn and the ribs were then immediately brought into apposition. A pre-loaded 1/2 cc syringe of tissue glue (VetBond™) was then used for closure.
The experiment to study emptying and absorption were performed at 1 and 2 weeks after this surgical procedure (cryo induced myocardial injury/infarction).

**Plasma BNP Measurement by Radioimmunoassay**

Plasma BNP levels were measured by radioimmunoassay (RIA). (Peninsula Laboratories, 5-2104 RIAS 9085). The assay is based upon the competition of $^{125}$I-labeled peptide and unlabeled peptide (unknown sample) binding to the limited quantity of antibodies specific for peptide in each reaction mixture. As the quantity of peptide in the unknown sample in the reaction increases, the amount of $^{125}$I-bound peptide able to bind to the antibody is decreased. By measuring the amount of $^{125}$I-bound peptide as a function of the concentration of the peptide in standard reaction mixtures, a "standard curve" is constructed from which the concentration of peptide in the unknown sample can be determined.

The summary of the assay protocol is as follows:

1- A 100 µl of unknown sample is pipetted in to duplicates glass test tubes

2- 100 µl primary antibody is added to unknown samples, vortexted, and incubated overnight at 4°C.
3- 100 µl of the I¹²⁵ labeled peptide is added and incubated overnight at 4°C.

4- Goat rabbit antiserum and normal rabbit serum is added and vortexed at 4°C and incubated for 90 minutes

5- 500 µl of RIA buffer is added, vortexed and centrifuged at 1700 x g for 20 minutes

6- The supernatant is aspirated off except in the total counts tube

7- Use gamma counter to count the level of radioactivity

8- The data obtained from the gamma counter is analyzed using the program Assayzap, Biosoft, GB, United Kingdom.

The detailed assay protocol is found in the product insert for the assay kit (Phoenix pharmaceuticals, C# RK-011-17)

The kit we used has a cross reactivity of 41% with the mouse BNP and total binding of 46%; calculations were made accordingly. The detection range for this kit was 10-128 pg/ml.

**Immunostaining for Non-Muscle Myosins**

Two WT mice were given a bolus of BNP at 10 ng/g body weight in 100 µl of modified Kreb’s solution followed by infusion of BNP at 1 ng/g for 30 minutes. One WT mouse was given 100 µl of vehicle and given an infusion of vehicle for 30 minutes.
1- The animals were then euthanized and intestinal tissue collected and sectioned into duodenum, jejunum, ileum and colon following anatomical demarcations.

2- The tissue is fixed in 4% paraformaldehyde over-night at 4°C.

3- The next morning the tissue is washed with phosphate buffered saline (PBS) for 15 minutes twice at room temperature with gentle rocking.

4- After the wash, the tissue is dehydrated and permeabilized as follows. Tissue is placed in ascending concentration of 30, 50 and 70 % methanol each for 15 minutes. Then the tissue is placed in a solution composed of 100 % methanol: DMSO: 30% \( \text{H}_2\text{O}_2 \) made at a ratio of 4:1:1. The tissue is left in this solution overnight at 4°C.

5- The next morning the tissue is washed in 70 % methanol for 30 minutes at room temperature with rocking.

6- Rehydration is accomplished by serially placing the tissue in 70 % methanol/PBS for 30 minutes with rocking, then 50 % of methanol/PBS with rocking, then 1 ml of PBS for 30 minutes with rocking, 1 ml of PBSMT (PBS, milk, Tween 20) 30 minutes with rocking twice.

7- The tissue is then incubated overnight with 1 ml of primary antibody diluted in PBSMT (1:250) with rocking at 4 °C.
8- The next morning the tissue is rinsed with PBSMT 2x with 1 ml for 1 hour at 4°C, 4 x in 1 ml for 1 hour each at room temperature.

9- Next the tissue is incubated overnight with 1 ml of the Secondary antibody diluted in PBSMT (1:250) at 4°C while rocking.

10- Next morning the tissue is washed as above with PBSMT

11- Next tissue is rinsed with PBT (PBS and Tween -20 equal concentration).

12- Post fixing in 4 % paraformaldehyde in PBS at 4 °C overnight.

13- Dehydration in the following sequence, 1 ml of PBT quick rinse, 1 ml PBT for 30 minutes at room temperature, 1 ml 50 % methanol for 30 minutes, 1 ml of 70 % methanol for 30 minutes at room temperature; 1 ml of 100 % methanol 30 minutes at room temperature twice.

14- Plastic embedding is achieved by transferring the tissue from 100 % methanol to araldite embedding medium and kept in medium for 3 hours. The tissue is then transferred into a fresh embedding medium into a mold on araldite rafts and the mold is kept overnight in 60° C oven to allow the araldite to harden.
The hardened plastic embedded tissue is then trimmed and sectioned using a microtome at 1-3 µm thickness. The sections pass automatically into water, the sections are transferred onto a slide and allowed to dry for 5-10 minutes on the surface of a hot plate. The first and last section are placed on one side and stained with toluidine blue to guide orientation.

Details of the above technique are found in (Linask & Tsuda, 2000).

**Statistical Methods**

We used a one way analysis of variance (ANOVA) to compare the average intragastric pressure at baseline, immediately after and later than five peptide half-lives after peptide injections. Fisher’s least significant difference (LSD) test is used as a post hoc test. Two sample t-tests were used to compare the measures of gastric emptying and absorption between peptide treated vs. vehicle treated mice. Specifically percent gastric emptying between treated vs. vehicle and average relative fluorescence units between the peptide treated vs. vehicle groups were tested using a two sample t-test.
CHAPTER THREE

THE EFFECT OF NATRIURETIC PEPTIDES ON INTRAGASTRIC PRESSURE, GASTRIC EMPTYING AND ABSOPRTION

INTRODUCTION

Measurement of gastric pressure using an intragastric balloon (manometry) offers the most direct way to quantify pressure changes inside the stomach (Malagelada & Stanghellini, 1985; Mearin & Malagelada, 1993). Other factors being constant, the measured gastric pressure is directly proportional to the force of contraction of the stomach wall (Mearin & Malagelada, 1993); therefore quantifying and comparing the rate of change in pressure between experimental groups yields a reliable and objective measure of gastric contractility. However, due to the inherent variability of baseline pressure among different animals, we further standardized the measurement by using the difference between the baseline and peak of the gastric contraction wave in our analysis. This makes our measurement less prone to variability and a more objective way to compare differences in
pressure between or among different groups of animals in these experiments.

Our measurements of gastric emptying and absorption were designed to be as close to the physiological state as possible. We used gavage feeding of fluorescence labeled dextran and let the mice roam freely for 30 minutes, following which the surgery was performed and measurement taken. The 70 kDa FITC-dextran was shown to be a valid measure of emptying in prior studies as was the 4 kDa FITC dextran for gastrointestinal absorption (Aube et al., 2006; Thorball, 1981). Since there is a theoretic possibility that there is a potential confounding by the action of BNP on arterial permeability and distribution of the dextran to the third space (rather than GI tract permeability or absorption) we measured and compared plasma fluorescence after intravenous injection of 4 kDa FITC-dextran in BNP treated and control animals. This avoids the potential confounding and validates our measurement.
RESULTS

Intragastric Pressure and Gastric Contractility

A typical intragastric pressure wave pattern is shown in figure 5; gastric contraction frequency averaged 3-7 times per minute ranging from 0.5 to 10 mmHg in amplitude.

Figure 5. Typical pressure wave pattern of a basal gastric contraction

A recording of a basal pressure pattern in windaq file is shown above. Division width = 4 seconds and division height =1 mmHg. Recording shown is a 10x compression (approximately 3 minutes) view of an actual experiment
Figure 6. Simultaneous recording of blood pressure and intragastric pressure

Two channel recording in our data acquisition system - windaq, Top panel shows blood pressure and bottom panel shows intragastric pressure. Note; division heights are 6.25 mmHg in top panel and 1 mmHg in bottom panel. Division width is 4 seconds in both channels. Recording shown is 10x compression of an actual experiment.
The prokinetic agent gherelin was used to ascertain that these recordings from the intragastric balloon corresponded to changes in gastric motility. Administration of gherelin resulted in a marked and significant increase in intragastric balloon pressure, validating that the contraction waves recorded are that of changes in gastric contractility. Gherelin was administered in three experiments and a typical response is shown in figure 7.

**Figure 7. The effect of intravenous administration of Gherelin on intragastric pressure**

Davison width = 8 seconds and division height = 1mmHg.
Arrow indicates point of injection of gherelin
Figure 8. Three individual experiments showing intragastric pressure after a 10 ng/g iv bolus of ANP, BNP and CNP.

Division height = 1mmHg. Division width = 300 seconds
As shown in figure 8, intragastric pressure was decreased after injection of any of the three natriuretic peptides, ANP, BNP or CNP and the pressure gradually returned towards the baseline values.

**Figure 9. Typical recording of post peptide injection gastric contraction wave pattern**

Gastric pressure was attenuated after injection of BNP without significant change in frequency of contraction. Also seen is a slight dip in mean arterial pressure following BNP injection. Arrow indicates point of injection. ‘Decompressed’ view, image shown is approximately 1 minute. A similar effect was observed for ANP and CNP.
Figure 10. The effect of C-ANP$_{4-23}$ on gastric pressure

Individual experiment showing no change in gastric pressure or blood pressure when the specific NPR-C ligand cANP$_{4-23}$ was injected in to wild type mice at 10 ng/g body weight iv.

Division width = 80 seconds
Figure 11. BNP did not change gastric pressure in NPR-A knock out mice

NPR-A knock out mice did not show decreased intragastric pressure with any of the natriuretic peptides. Here BNP was administered at 10 ng/g body weight iv.

We quantified the change in intragastric pressure by averaging the difference between the peak and baseline gastric pressures for three thirty minute periods; before, immediately after and later than 5 peptide half-lives after peptide injection.
Since each experimental animal had different baselines; comparing the difference between baseline and peak (i.e., the change in amplitude of the gastric contraction wave) was found to be more precise and reliably comparable among different experimental animals. The basal pressure was defined as the lowest pressure immediately before a peak and the peak was determined by moving the cursor and recording the highest point of the peak pressure.
Figure 13 (below) shows the pooled data on comparison of the average gastric pressure before peptide injection (Basal), immediately following peptide injection and later than 5 BNP half-lives (Recovery) vs. vehicle. As shown, BNP significantly decreased intragastric pressure from a basal value of 2.26 ± 0.29 mmHg to 1.44 ± 0.11 mmHg and gastric pressure returned to 2.08 ± 0.17 mmHg when measured later than 5 BNP half-lives (n=5, p<0.05, ANOVA, Fisher’s LSD test). Similar and statistically significant reduction of gastric pressure was obtained for ANP and CNP (Figures 14 and 15). ANP significantly decreased gastric pressure from a basal value of 2.11 ± 0.3 mmHg to 0.7 ± 0.25 mmHg and CNP decreased gastric pressure from a basal value of 1.91 ± 0.3 mmHg to 0.85 ± 0.24 mmHg. Average gastric contractions per minute were 4.4 ± 0.7, 3.1 ± 0.4 and 4.3 ± 0.5 for periods of BNP injection compared to 5.3 ± 1.2, 4.2 ± 0.6 and 4.3 ± 0.5 for the vehicle group (all p>0.05, ANOVA). There was no difference in gastric pressure in BNP treated vs. vehicle treated NPR-A KO mice.
Mean reduction in intragastric pressure (measured in mmHg) following intravenous BNP 10 ng/g in 100 µl of vehicle vs. 100 µl of vehicle injection to WT mice (n = 5 in each group). Intragastric pressure measured before (Basal), immediately after (Post Injection) and more than 5 peptide half-lives after injection (Recovery) is shown.

BNP significantly (* = p < 0.05, ANOVA, Fisher’s LSD test) decreased intragastric pressure compared to vehicle. Gastric pressure returned toward basal levels when measured later than 5 BNP half-lives.
Mean reduction in intragastric pressure (measured in mmHg) following intravenous ANP 10 ng/g in 100 µl of vehicle vs. 100 µl of vehicle injection to WT mice (n = 5 in each group). Intragastric pressure measured before (Basal), immediately after (Post Injection) and more than 5 peptide half-lives after injection (Recovery) is shown.

ANP significantly (* = p < 0.05, ANOVA, Fisher’s LSD test) decreased intragastric pressure compared to vehicle. Gastric pressure returned toward basal levels when measured later than 5 ANP half-lives.
Figure 15. Mean reduction in intragastric pressure following intravenous CNP given at a dose of 10 ng/g body weight

Mean reduction in intragastric pressure (measured in mmHg) following intravenous CNP 10 ng/g in 100 µl of vehicle vs. 100 µl of vehicle injection to WT mice (n = 5 in each group). Intragastric pressure measured before (Basal), immediately after (Post Injection) and more than 5 peptide half-lives after injection (Recovery) is shown.

CNP significantly (* = p < 0.05, ANOVA, Fisher’s LSD test) decreased intragastric pressure compared to vehicle. Gastric pressure returned toward basal levels when measured later than 5 CNP half-lives.
**Gastric Emptying**

Gastric emptying was measured by gavage feeding a 0.1 ml of 2.5 mmol 70 kDa FITC labeled dextran to conscious mice immediately after a 10 ng/g body weight bolus of BNP. The mice were sacrificed 30 minutes after gavage and the amount of fluoresce that has emptied the stomach as a percent of total fluorescence measured in the entire GI tract was calculated and compared between the BNP treated vs. vehicle treated mice.

**Figure 16. The effect of BNP on gastric emptying**

Percent gastric emptying, measured in amount of fluorescence that emptied the stomach as a percentage of the total fluorescence measured in the entire gastrointestinal tract 30 minutes after gavage feeding of 0.01 ml of 2.5 mmol 70 kDa FITC-dextran.

BNP (10 ng/g iv) significantly decreased gastric emptying in wild type mice compared to vehicle (n =5, p < 0.05, t-test,). This effect of BNP was absent in NPR-A knockout mice (n = 5, p > 0.05, t-test).
Figure 17 shows percent decrease in gastric emptying as a function of BNP dose in WT mice. Measurements were taken 30 minutes after gavage feeding of 0.01 ml/g of 2.5 mmol FITC-dextran. Progressive doses of BNP at 5, 10 and 100 ng/g iv resulted in significant reduction of gastric emptying. (p < 0.05, n = 4 in each group, ANOVA, Fisher’s LSD test)
Absorption

To measure absorption we used gavage feeding of 22ml/kg of a solution containing 22mg/ml 4 kDa FITC-dextran to conscious mice immediately following a 10 ng/g dose of BNP vs. vehicle. The plasma fluorescence (taken 1 hour after gavage) was measured and compared between the two groups. Plasma fluorescence after iv injection of FITC dextran is also measured and shown below to ascertain that the difference was not due to distribution rather than absorption.

Figure 18. The effect of BNP on absorption

Absorption measured in relative plasma fluorescence units one hour after gavage feeding of 4 kDa FITC-dextran in wild type mice BNP treated (10 ng/g iv) vs. vehicle (n = 5, p < 0.05, t-test). No significant difference in absorption was observed between BNP vs. vehicle treated NPR-A KO mice (p > 0.05).
Figure 19. Plasma fluorescence following intravenous FITC-dextran, BNP vs. Vehicle

Relative fluorescence of a 50µl plasma sample taken 1 hour after intravenous administration of 100 µl 0.5mmol 4kDa FITC-dextran is shown. BNP treated (10 ng/g iv) vs. vehicle in wild type mice, (p > 0.05, n = 4 in each groups, t-test).
Mean arterial blood pressure was continuously monitored during all experiments using an intra-carotid catheter. There was a slight reduction of average blood pressure from 62.9 ± 4.7 mmHg prior to BNP injection to 59 ± 3.2 mmHg (n=5, p>0.05, ANOVA, Fisher’s LSD test). Blood pressure returned to 61.4 ± 5.08 mmHg when measured later than 5 peptide half-lives.

Similar drops in blood pressure were also observed during the first few minutes after injection of both ANP and CNP with return of blood pressure towards baseline.

Plasma BNP levels averaged 4500 pg/ml and the levels fell to 725 pg/ml at 30 minutes post injection and to undetectable levels at 90 minutes post injection.
DISCUSSION

These experiments show that the natriuretic peptides tested, namely ANP, BNP and CNP all decreases intragastric pressure (gastric contractility) in anesthetized mice. Prior studies have shown that natriuretic peptides decrease contractility of isolated gastric and intestinal smooth muscle cells in vitro (GuoCui et al., 2003; Yasuda et al., 2000), this study is the first to show this effect to be true in the whole intact animal. Furthermore, the inhibitory effects of the natriuretic peptides appears to be dose dependent and mediated primarily by NPR-A.

We further investigated whether this effect on contractility has a functional significance in the whole animal. To accomplish this we focused the subsequent studies on the effect of BNP on gastric emptying and absorption; with its increasing utility in the diagnosis and treatment of heart failure, further characterization of an aspect of BNP is deemed to be potentially of important translational benefit.

Our findings confirmed that this effect of BNP on contractility is accompanied by significant reduction in gastric emptying and gastrointestinal absorption; clearly demonstrating that this inhibitory effect on the gastrointestinal tract has functional significance in the whole animal.
As shown in Figure 19, plasma fluorescence one hour after iv administration of 4 kDa FITC-dextran was similar in BNP treated vs. vehicle. This finding further strengthens our conclusion that the reduced plasma fluorescence in gavage fed and BNP treated mice was indeed due to decreased absorption or permeability in the GI tract and not due to other known actions of BNP such as increased renal excretion or change in vascular redistribution (Huxley et al., 1987).

Although there has been some indication that natriuretic peptides are involved in inhibitory regulation of GI function (Ebert, 1988; Olsson & Holmgren, 2001), our findings are the first to document a specific inhibitory role for BNP on emptying and absorptive functions in the GI tract.

The average plasma BNP levels immediately after injection averaged 4500 pg/ml. While this level is supra-physiological, it is not uncommon in patients with heart failure (Fitzgerald et al., 2005). Furthermore, even much higher levels of BNP are consistently seen when recombinant BNP (Nesiritide) is administered for the treatment of heart failure (Colucci et al., 2000). Moreover, the BNP level in our experiments fell to levels routinely seen in heart failure patients at 30 minutes post injection. Therefore, our experimental model provides novel insight into what would be expected in heart failure or when BNP is exogenously administered.
Natriuretic peptides bind to transmembrane receptors that have guanylyl cyclase (GC) activity. ANP and BNP bind to NPR-A where as CNP binds to NPR-B. The ensuing peptide receptor interaction increases intracellular cGMP with subsequent enzymatic steps that regulate cellular functions in various tissues where these peptides and/or receptors are expressed (Kuhn, 2005).

In our study, NPR-A KO mice did not show a significant response with any of the peptides we used (ANP, BNP, CNP or c-ANP4-23). This suggests that; the gastrointestinal effects of BNP and the other natriuretic peptides are likely to be specifically mediated by the NPR-A receptor. Since c-ANP4-23 specifically binds to NPR-C (Anand-Srivastava, 2005), the absence of a GI effect when c-ANP4-23 was injected into WT or NPR-A KO mice is additional evidence that NPR-A may be the major receptor mediating the effect of BNP on gastric emptying and absorption. Our finding is consistent with previous studies that have shown that the inhibitory effect of CNP on isolated gastric smooth muscle cells is mediated by a cGMP-dependent pathway (GuoCai et al., 2003; Scotland et al., 2005).

The natriuretic peptides in general are among some of the most evolutionarily conserved peptides across many species of the phylogenetic tree with various functions in fluid homeostasis. Studies done early in the discovery of natriuretic peptides have reported that
ANP significantly decreased jejunal fluid absorption in dogs and rats (Morita et al., 1992; Scott & Maric, 1991). More recent studies have shown that natriuretic peptides cause upregulation of aquaporin 3 expressions in human colonic epithelia signifying their potential role in fluid homeostasis (Pacha, 2000). ANP has also been shown to be important in promoting sea water adaptation in eels and decreases intestinal sodium absorption (Tsukada et al., 2005). However, the great majority of recent studies done on BNP have focused on its role in modulating blood pressure, diuresis and natriuresis. Since the primary stimulus for release of ANP and BNP is mechanical stretch of the atrial and ventricular myocardium, their expression and release is closely linked to body fluid volume status (Cowie & Mendez, 2002; James et al., 2005). Moreover, the temporal pattern of expression and release of BNP following a given stimulus, such as an acute myocardial infarction, indicates that the endocrine heart could potentially employ varying plasma levels of the natriuretic peptides to modulate body fluid volume (Silver, 2006). Since natriuretic peptide receptors are expressed on the gastric and intestinal smooth muscle cells, and we show that intravenously administered BNP decreased gastric emptying and absorption, it is logical to deduce that our finding may be an indication that the endocrine heart employs natriuretic peptides to delay or modulate the rate and amount of water and solute absorption.
from the gastrointestinal tract. From a physiological standpoint the effect of cardiac hormones on absorption in the GI tract is a beneficial extension of their role in volume homeostasis. Theoretically, such a role could extend to pathophysiological states such as heart failure where plasma BNP levels and volume overload progressively rise (Barclay et al., 2006) and modulation of volume status becomes even more critical for survival.

We have shown that high plasma levels of BNP (sustained levels of 500 pg/ml or greater), significantly decrease gastric motility, emptying and absorption in mice. This appears to be a common effect of the natriuretic peptides shared by ANP and CNP. The absence of this GI effect in receptor knockout mice and the dose-response relationship suggests a receptor mediated specific event.

While our study in the mouse model can not be generalized to humans, our findings that BNP significantly decreased gastric emptying and absorption offers valuable new insights into the role of the gastrointestinal tract in fluid homeostasis, especially during heart failure. First, symptoms of perturbed gastrointestinal function such as nausea, dyspepsia, indigestion and malabsorption are frequently seen in patients with heart failure where plasma BNP levels are markedly elevated (Krack et al., 2005; Shamsham & Mitchell, 2000). Our study suggests that some of these symptoms could at least partly be
attributable to the elevated BNP. Secondly, such an effect by BNP could potentially add a new area of interest and investigation in the role of the heart as an endocrine organ. While there are established physiological and pathophysiological cardio-renal regulatory pathways; a possible 'cardio-gastric and/or cardio-intestinal' link via natriuretic peptides appears to be another possible pathway involving the endocrine heart.
CHAPTER FOUR

THE ROLE OF BNP IN THE GASTROINTESTINAL MANIFESTATION OF MYOCARDIAL INJURY

INTRODUCTION

Prior studies have utilized myocardial injury models to study pathophysiological and histological effects exerted by the natriuretic peptides on the cardiovascular system. Based on the results of our experiments reported in the earlier chapters, mainly the fact that the natriuretic peptides in general and BNP in particular decrease gastric emptying and absorption; we postulated that these peptides would have a similar effect in the face of acute myocardial infarction.

The most commonly used methods of inducing experimental ischemia or myocardial infarction (MI) in mice are the permanent ligation of left anterior descending (LAD) coronary artery and the Cryoinfarction (freeze-thaw) method. In LAD ligation, the artery is tied with surgical sutures whereas in cryoinfarction a blunt frozen metallic probe is directly applied to a specific area of the myocardium with resulting thermal ischemia at the point of contact and surrounding
myocardium. Although the cryoinfarction method was introduced as early as 1948 (Hass & Taylor, 1948) it is only recently that it is shown to have several advantages over LAD ligation; as the LAD method is associated with marked variability in the size of the infarct and leads to apical infarct resulting in ventricular aneurysm (van den Bos et al., 2005). In the cryoinjury model the infarct area is limited to the anterior wall of the myocardium more closely resembling what is encountered in clinical practice in humans; where reperfusion therapy results in limited infarct size and chances of developing apical aneurysm are becoming increasingly less likely (Huwer et al., 1998; Roell et al., 2002; van den Bos et al., 2005). Pathophysiologically, The cryoinfarction method causes acute cell death probably from the mechanical process associated with thermal injury; the infarct border therefore corresponds to the size of the probe hence the improved consistency of the resulting infarction as compared to the LAD ligation method (van den Bos et al., 2005). Moreover since the cryoinfarction method has a much lower peri and post operative mortality, fewer numbers of animals will be needed for a given study.

We used a cryoinfarction model as the degree and distribution of the infarct (Cell death and fibrosis) is more consistent with the cryoinfarction as compared to the Left anterior descending artery (LAD) ligation method (Huwer et al., 1998; Roell et al., 2002).
This study was designed to test whether a cryoinfarction of the myocardium leads to change in gastric emptying and absorption in mice and whether the difference in the plasma BNP levels between the MI and Sham mice is responsible for this difference. The study was also done in NPR-A knock out mice to test whether this effect is mediated by NPR-A as shown by our previous experiments.
RESULTS

As shown in figure 20, our cryoinfarction model produced myocardial cell death and fibrosis similar to what will be seen in myocardial infarction. The infarction was limited to the area under the application of the frozen probe and immediate surrounding myocardial tissue without extension to the ventricular apex. As a result no ventricular aneurysms were observed in our model.

We tested and compared the degree of gastric emptying at one and two week after infarction to establish the differences in plasma BNP levels between the MI and sham mice. Subsequent experiments on absorption were done at two weeks after infarction.

When gastric emptying was measured one week after infarction, it was significantly decreased in the cryoinfarction group as compared to the sham. Percent gastric emptying was 67.5% ± 5.8 for the MI group vs. 88.7% ± 2.9 for the sham group (P<0.05, t-test) as shown in figure 21. The plasma BNP levels were elevated in both groups but significantly higher in the MI group as compared to the sham group.

BNP levels were 4292.2 ± 276.5 1 week after MI vs. 105.4 ± 11.3 in sham mice (n = 5, p<0.05, t test). BNP levels were 1964.7 ± 755 two weeks after MI, (n=5, p<0.05, t test compared to one week post MI).
Figure 20. Representative histological appearance of the myocardium 14 days after cryoinfarction. Stain: Hematoxylin & Eosin 4x (Top); 20x of the boxed part (Bottom)
Figure 21. Comparison of percent gastric emptying in sham vs. MI in Wild Type mice; one week after cryoinfarction

WT mice, 67.5% ± 5.8% for mice with MI vs. 88.7± 2.9 % for sham (n=7, P<0.05)
Figure 22. Comparison of percent gastric emptying in sham vs. MI Wild Type mice, two weeks after cryoinfarction.

WT mice, 82.2% ± 0.5% for mice with MI vs. 97.9 ± 0.4 % for sham (n=5, P<0.05) P= 0.017.

As shown in figure 22 above, two weeks after infarction, percent gastric emptying values were also significantly lower in the MI group as compared to the sham group 82.2% ± 0.5% for MI vs. 97.9 ± 0.4 % for sham (n=5, P<0.05).
Figure 23. Comparison of Percent gastric emptying in sham vs. MI NPR-A KO mice, two weeks after cryoinfarction

KO mice, 84.6% ± 0.7% for mice with MI vs. 87.6 ± 054 % for sham (n=6, P >0.05) P= 0.07

In NPR-A knock out mice, percent gastric emptying was identical between the MI vs. the sham group 84.6% ± 0.7% for mice with MI vs. 87.6 ± 054 % for sham (n=6, P >0.05).
There was also a statically significant difference in the degree of absorption measured in relative fluorescence units (RFU) in the plasma following a gavage meal containing 22ml/kg of 22mg/ml 4 kDa FITC dextran. In wild type mice, absorption was, 631.9 ± 121 (RFU) for the MI group vs. 349.8 ± 78.6 (RFU) for the sham group; n=6, P< 0.05.

**Figure 24. Comparison of absorption measured in relative Plasma fluorescence units. Sham vs. MI, WT mice**

Absorption measured in Relative plasma fluorescence units

WT mice, 631.9 ± 121 for mice with MI vs. 349.8 ± 78.6 for sham n=6, P< 0.05 (P=0.04)
In NPR-A knockout mice the difference in absorption was not statistically significant. Absorption measured in Relative plasma fluorescence units in NPR-A KO mice was, 516.2 ± 107.3 for mice with MI vs. 366.5 ± 39 for sham n=6, P> 0.05  (P=0.1).

Figure 25. Comparison of absorption measured in relative plasma fluorescence units. Sham vs. MI, NPR-A KO

Absorption measured in Relative plasma fluorescence units NPR-A KO mice, 516.2 ± 107.3 for mice with MI vs. 366.5 ± 39 for sham n=6, P> 0.05  (P=0.1).
The presence of dyspeptic symptoms in heart failure and during acute myocardial ischemia has been known for a long time. Studies done as early as 1966 have reported what was then described as venostatic gastritis where venous congestion is believed to be the cause of gastric pathology (Fixa et al., 1966). The presence of nausea, vomiting and indigestion during acute myocardial infarction and in the course of heart failure has also been extensively reported in the literature (Abrahamsson & Thorén, 1973; Ahmed et al., 1978; Camurça et al., 2004; Pasini et al., 1989; Wei 1988). Studies that have looked into mechanisms of such an association have in the past mainly pointed to a possible chemoreceptive or neurally mediated reflex known as the Bezold-Jarish reflex (Chianca et al., 1997; Sleight, 1981).

Both ANP and BNP have previously been shown to have cardioprotective effect during acute myocardial infarction. For instance, the effect of ANP and BNP on renal salt handling was shown to be specifically enhanced during acute myocardial ischemia irrespective of the level of activation of the renin angiotensin aldosterone (RAS) system (Charles et al., 2003; Rademaker et al., 2000). ANP was also shown to have important volume regulation role
during acute heart failure induced by ventricular pacing (Lee et al., 1989). Both BNP and ANP are also shown to have an inhibitory effect on regional sympathetic activity in the kidney and the heart (Brunner-La Rocca et al., 2001). While the pathways that lead to sympathetic activity are not clearly understood, the end result of decreased sympathetic activity is cardioprotective. Studies that looked at the receptors involved in such processes have consistently shown that these effects are mediated by cGMP coupled pathways. While some of this effect is indirectly mediated via the renin angiotensin aldosterone (RAS) system, protective actions of guanylyl cyclase-A that are not mediated by the RAS have also been shown (Li et al., 2002; Nakanishi et al., 2005).

In our experiments, we tested the effect of acute myocardial injury on gastric emptying at one and two weeks post infarction and compared the results along with the plasma BNP levels. There was a significant reduction in gastric emptying at one week in mice with myocardial infarction compared to sham. It is interesting to note that even the sham mice had a slightly lower rate of gastric emptying at one week compared to controls (baseline values). As the BNP levels were higher in sham mice than in controls, and the levels were markedly higher in the infarcted mice than the sham mice, this is an indication that BNP levels corresponded with the degree of gastric
emptying. At two weeks post infarction, the sham mice had gastric emptying statistically identical to controls where as infarcted mice continue to show a markedly lower rate of gastric emptying, this again indicates that the BNP levels significantly correspond with the degree of gastric emptying adding evidence that BNP may be the major (or one of the major) reasons for the observed differences. Since our data shows that infarcted receptor knock out mice had no significant difference in gastric emptying compared to sham, this validates our assertion that the BNP difference played a role in the observed effect and this effect is probably mediated through the NPR-A receptor.

Our absorption data for wild type mice is consistent with our central hypothesis and infarcted mice did show significantly lower absorption rate as compared to sham. The data in the knock out mice in our experiment is less conclusive, since the knock out mice with MI did show a reduced absorption compared to the sham mice. There are several explanations for this finding. First of all, myocardial injury is known to activate a series of systems other than the natriuretic peptides, and secondly the process of absorption in the GI tract is also likely to involve several different mechanisms. For instance the renin angiotensin aldosterone system is known to be activated with myocardial injury, inflammatory mediators such as tissue growth factor β and tumor necrosis factor are also a few of the cytokines that
are released from infarcted myocardium and circulate in the plasma with potential effects in the GI tract. Therefore the observed effects in NPR-A knock out mice may be manifestations of activation of these systems that would be expected to be intact in the NPR-A knock out mice.

It has also been shown that intravenous volume expansion decreased gastric emptying and permeability of the mucosa to water and solutes and vagus nerve mediated neural mechanism were postulated as mechanisms for such an effect (Chang EB and Rao MC., 1994). It was also reported that acute blood volume expansion with intravenous fluids decreased net sodium absorption in the jejunum of rats and dogs (Duffy et al., 1978; Richet & Hornych, 1969). Moreover experiments in human volunteers have shown that body position changes such as recumbency and simulation of hemorrhage resulted in significant increase in intestinal water and salt absorption (Sjövall et al., 1986). Despite this known relationship between gut motility, gastric emptying and intestinal absorption on one hand and intravenous venous expansion and contraction on the other, most of the studies that have investigated this relationship were limited to the vagal or sympathetic nervous system. Nevertheless, with the knowledge base accumulated in the field of natriuretic peptides over the past two decades, it is only logical to conjecture that at least some
of the changes observed in gastric emptying and intestinal absorption
during acute MI or heart failure may be due to natriuretic peptides.

The data supporting the relationship between volume expansion
and gastric emptying and intestinal water and salt absorption is fairly
strong. What was missing was an experiment to directly and
specifically test whether the elevated natriuretic peptide levels (caused
by volume expansion or myocardial injury) corresponded with gastric
contractility, gastric emptying and absorption. Together with our
earlier findings that all the natriuretic peptides significantly decrease
gastric contractility, our data on BNP showing significant reduction of
gastric emptying and absorption; the confirmation of these findings in
an acute myocardial infarction model is a strong indication that plasma
BNP (and natriuretic peptide) elevation during acute MI and / or heart
failure is at least partly responsible for these observed effects. More
importantly, the previously confirmed effect of increased plasma
volume on gastric emptying and absorption is validated by our data to
be mediated by natriuretic peptides in general and by BNP in
particular. This finding strengthens the case for a humoral link
between the heart and the GI tract and opens up new avenues for
heart failure research. We believe further research in this area could
identify potential new targets for the treatment of heart failure.
CHAPTER FIVE

BNP AND NON MUSCLE MYOSINS IN THE GASTROINTESTINAL TRACT

INTRODUCTION

Myosins are proteins that are involved in mechanical force generation and transduction. The characteristic myosin molecule is composed of two heavy chain subunits measuring approximately 200 kDa each, which form a globular amino-terminal head region, and a coiled carboxyl-terminal tail. The globular head region is non-covalently associated with two pairs of light chains of 20 and 17 kDa (Kelley & Adelstein, 1990). Typically, myosins interact with another protein; actin, in a process that involves the hydrolysis of ATP. Classically such actomyosin interaction is discussed in the context of muscle contraction and relaxation. However myosins are also expressed in non muscle tissue (Sellers, 2000).
In non muscle cells, the prototype non muscle myosin type-II regulates actin organization into filaments and its functions include maintaining the cell shape, cell division and cell movement (Bresnick, 1999). Non muscle myosin-II is also involved in epithelial cell attachment at the tight junction regulating paracellular permeability (Hecht et al., 1996). Tight junctions are integral part of the intestinal villi absorptive structures.
**Intestinal Villi**

*Figure 27. Schematic depiction and electron micrograph of the structures of intestinal villi*

Adapted from Keith R. Porter and S Clark

The electron micrograph (above) shows the microvilli of a mouse intestinal cell. Incorporated in the plasma membrane of the microvilli are a number of enzymes, mucous producing goblet cells and endocrine / paracrine cells that produce a number of hormones, among which are the natriuretic peptides and guanylin.
Figure 28. Cytoskeletal structures of the intestinal microvillus.

Adapted from Ross, Histology: A Text and Atlas, 4th ed.
Figure 29. Ultra structures of the microvilli cytoskeleton

Adapted from (Hirokawa & Heuser, 1981)

Actin cytoskeleton is shown by the arrow(s) in panel A, and in panel B the quick freeze, deep etch, rotary replication images show the core of the microvilli and its attachment at the cytoskeleton base in the cytoplasm
Figure 30. Transmission Electron micrograph of intestinal villi in various state of contraction

Chicken intestinal epithelia showing increasing state of contraction (top to bottom). Note ‘fanning’ of the microvilli as the degree of contraction increase. Transmission electron micrograph. Top; 6100x, middle; 5600 x and bottom 8900x.

Adapted from (Burgess, 1982)
**Tight Junctions**

Tight junctions seal adjacent epithelial cells in a narrow band just beneath their apical surface. They perform vital functions including regulation of the passage of molecules and ions through the space between cells and blocking of movement of the integral membrane proteins between the apical and basolateral surfaces.

**Figure 31. Schematic depiction of tight junctions**

Studies done in kidney, bladder and intestinal epithelia have confirmed changes in permeability regulated by phosphorylation related contractile changes at the tight junction. These changes primarily increase paracellular movement of water and small molecular weight solutes (Broschat et al., 1983; Hecht et al., 1996; Keller & Mooseker, 1982; Swanljung-Collins & Collins, 1992). Non muscle myosin type IIB is phosphorylated...
by casein kinase-II and dephosphorylated by a phosphatase that is regulated through the action of Rho–associated kinase (Li & Gorodeski, 2006).

Since the physiological effects of the natriuretic peptides are mediated via cGMP dependant protein kinases, we found it intriguing to examine whether treatment with BNP would reveal changes in the distribution of non muscle myosins and contractility pattern of the intestinal villi and thus shed some light on the mechanism for the observed effect of BNP on absorption.

We treated wild type mice with a 10 ng/g iv bolus of BNP followed by a 1ng/minute infusion for 30 minutes. After the 30 minute infusion the mice were sacrificed and intestinal tissue removed and sectioned into, jejunum, ileum and colon and immunostained for non muscle myosin as described in the methods section. We used the vehicle as an experimental control. We also used a tissue section not treated with first antibody as a methodological control to evaluate the potential confounding by non specific binding.
RESULTS

Figure 32. Toluidine blue stained 3µm sections of a mouse intestinal tissues

Cross sections of intestinal tissue from our experiments are shown. Top panel, Jejunum; middle panel, ileum; and bottom panel, colon.
Images shown are 3 µm section of Jejunal villi (top) and Ileum (bottom), stained with toluidine blue. Magnification is 40x top, and 20x bottom.
Figure 34. Electron micrograph of the intestinal microvilli

Control vs. BNP treated

Control, 20 000x  BNP treated, 20 000x

Microvilli in BNP treated mice show loss of the distinctive 'fanning' of the microvilli that is typical of markedly contracted state.
Figure 35. Jejunal villi immunostained for non muscle

myosin type IIB - Control

Longitudinal section at 20x (Top) and cross section 40x bottom
Figure 36. Comparative images of control vs. BNP treated jejunal villi

Images show jejunal villi, immunostained for non muscle myosin type IIB. Control (TOP) and BNP treated (bottom). Note the marked increase in fluorescence in BNP treated as compared to control. Immunofluorescence, 20 x magnification.
Figure 37. Jejunal villi and smooth muscle bundles immunostained for non muscle myosin

Cross section view of the mouse ileum is shown. Note the circular and longitudinal smooth muscle bundles with distinct stain (fluorescence) for non muscle myosin type IIB. The core of the villi also shows the distinct staining pattern.

Magnification, 10 x
Figure 38. A 3 µm sections of a mouse jejunum immunostained for non muscle myosin type IIB. Control vs. BNP

Control top and BNP treated bottom. Note the markedly increased staining of the villus core and the crypt in BNP treated mouse. 20x
DISCUSSION

Structures necessary for force generation and mechanotransduction such as actin and myosin and their associated proteins are integral components of the cytoskeleton of the intestinal villi (Mooseker, 1976; Mooseker et al., 1983; Rodewald et al., 1976; Rostgaard & Thuneberg, 1972). Although the mechanism of contraction of intestinal villi still remains unsettled, different studies have reported mechanisms of contraction that are both ATP and calcium dependent and others reporting calcium independent mechanisms of contraction (Burgess, 1982; Mooseker, 1976; Swanljung-Collins & Collins, 1991). Still other studies have reported contraction at the tight junctions but not of the microvilli themselves (Keller & Mooseker, 1982).

Our electron micrographic observations indicate that elevation of BNP in the plasma appears to cause the microvilli to lose the typical ‘fanning’ or separated appearance that is characteristic of contraction at the crypt region of the intestinal microvilli. Such appearance was previously shown to be due to contraction at the tight junction regions involving a circumferential contractile ring (Burgess, 1982). Tight junction
contraction in turn results in increased paracellular permeability (Hecht et al., 1996; Turner et al., 1997). Therefore our electron micrographic observation (decreased contraction of the tight junction contractile ring) supports the hypothesis that elevated BNP in the plasma makes the tight junctions of intestinal villi less permeable to water and small molecular weight solutes. Our fluorescence immunostaining images also support this hypothesis as we show enhanced distribution of non muscle myosins along the crypts of the villi; which may be an indication that the enhanced distribution corresponds to a change in tautness of the tight junctions thereby decreasing paracellular movement while allowing apical transport of nutrients. Apical transport of electrolytes and nutrients (sodium and glucose for example) is mediated by the sodium glucose cotransporter (SGLT1) and is accompanied by increased tight junction permeability to small solutes (Turner et al., 1997). Physiologically such coordination between the apical and basolateral surfaces of the villus would be advantageous; as it allows regulated (transporter mediated) absorption across the apical membrane while directing water and small solutes to the tight junction. However whenever there is a need to decreases volume, as in the case of heart failure; decreasing free
paracellular movement of water while still permitting apical transport of nutrients would allow for a more homeostatic absorptive function; especially when modulating volume becomes essential as in the case of heart failure. Utilizing BNP and possibly the other natriuretic peptides for this process would have physiological benefit for the heart; after all the heart will be ‘interested’ in getting all the nutrients it can get during heart failure while delaying or decreasing absorption of water and salt that could add to its stress.

Although the effect of BNP and natriuretic peptides in general on permeability of intestinal epithelia has not been extensively studied in the past, it is known that natriuretic peptides in general have an effect on permeability of the vascular epithelium (He et al., 1998; Huxley et al., 1987; Kubes, 1993; Lofton et al., 1990). Studies that have looked into this effect of ANP and BNP on permeability of pulmonary vascular epithelium have shown that these effects were at least partially reproduced by cGMP or cGMP analogs (Draijer et al., 1995; Hölschermann et al., 1997; Klinger et al., 2006; Westendorp et al., 1994). A more direct effect that is independent of cGMP was also shown for ANP (Kubes, 1993). Similarly, other studies that have investigated the effect of ANP on counteracting the
endothelial permeability and inflammatory effect of tumor necrosis factor (TNF) have shown that this effect is mediated by NPR-A and cGMP and it is targeted at cytoskeletal actin organization (Kiemer et al., 2002). An other support for this finding comes from the observation that vascular endothelial growth factor (VEGF) is inhibited by ANP (Pedram et al., 2002). VEGF is also known as vascular permeability factor and is involved in stabilization of actin association with tight junctions; and this function was reversed by guanylyl cyclase receptor antagonists (Pedram et al., 2002). The precise mechanism of this process is still unclear, some studies indicated it involves heat shock protein 27 (HSP27); a protein with known functions in actin organization and whose actions are mediated by cGMP dependent protein kinase (Butt et al., 2001). Other studies suggest mechanisms that involve Phosphorylation of structural and contractile proteins; including actin and myosin (Hecht et al., 1996; Ma et al., 2000; Turner et al., 1997), Specifically at the prejunctional ring of actin and myosin; sites that are characteristic of contractile function at the tight junction (Madara et al., 1987).

Our immunofluorescence images revealed an enhanced distribution of non muscle myosins in the intestinal villi core and
along the crypts (tight junctions). While we haven’t done biochemical analysis to correspond with these findings, previous studies have shown that phosphorylation related proteins such as mitogen-activated protein (MAP) kinase and Rho of the ras family of proteins are associated with tight junctions and this in turn controls tight junction contractility (Murakami et al., 1994; Zahraoui et al., 1994). Interestingly, both ANP and BNP are known to exert anti-inflammatory (thus decreased vascular endothelium permeability) effects in vascular endothelium through mechanisms that involve MAP-kinase and MAP kinase phosphatase-1 (MKP-1) (Fürst et al., 2005; Weber et al., 2003).

Although more research needs to be done in this direction, our electron micrographic imaging and immunostaining data coupled with what is previously known about epithelial tight junction function lends enough support to our hypothesis and helps formulate a basis for future direction. The finding that immunostaining of non muscle myosin in BNP treated mouse tissue shows enhanced distribution of non muscle myosins along the crypts and core of the intestinal microvillus appears to be a recurring theme. Tight junctions appear to contract less and microvilli assume the typical non-contracting appearance when BNP plasma level was raised. These findings are associated with
decreased paracellular permeability at least in the case of the vascular endothelium. Whether the biochemical signaling mechanisms known for vascular endothelium will be the same in the intestinal villi and whether it will have similar physiological consequences remains to be investigated.
CHAPTER SIX
SUMMARY AND CONCLUSIONS

The main objective of my study was to show that there indeed is a humoral link between the heart and the gastrointestinal system. This humoral link appears to utilize natriuretic peptides although it is possible that there probably are several other as yet undiscovered peptides or even other molecules that serve this same purpose.

To this aim, we demonstrated that ANP, BNP and CNP decreased contractility of gastric smooth muscle in the whole intact animal. Confirming the previously done \textit{in vitro} experiments that showed natriuretic peptides decreased contractility of isolated gastric and intestinal smooth muscle cells. This effect was shown to have a functional significance in that; these experiments showed that gastric emptying and absorption from the gut were decreased in the whole animal when BNP was intravenously administered. We also presented data showing myocardial injury and the associated elevation in plasma BNP levels decreased gastric emptying and absorption from the GI tract. Although the obvious reason for this result may be the effect of BNP on smooth muscle relaxation, we also show data supporting the
hypothesis that BNP and natriuretic peptides have a more direct effect on absorptive structures of the intestinal villi possibly affecting paracellular transport of water and electrolytes.

In summary one can garner the following observations from these experiments

a) Natriuretic peptides decrease gastric contractility and gastric emptying in intact whole animals

b) BNP decreased gastric emptying in a dose dependant manner

c) BNP decreased absorption from the GI tract when administered at dose designed to raise the plasma level to the levels seen in heart failure

d) Myocardial injury and elevation of BNP in the plasma was associated with decreased gastric emptying and absorption

e) Elevated plasma BNP has en effect on contractile structures of the intestinal villi and this effect appears to be directed at decreasing paracellular movement of water and solutes
PERSPECTIVES

The next line of research should follow the intriguing questions raised by our immunofluorescence data. How and why does the distribution of myosin in the microvilli appear to be increased by BNP? What are the molecular mechanisms that are involved here? Is the electron micrograph appearance of microvilli indication of contraction at the tight junction? Is paracellular movement of water and solutes decreased by BNP as suggested by our imaging data? Are there other transporters that direct water and sodium absorption to the basal and apical membranes; if so can they be therapeutically targeted to regulate sodium absorption from the gut? Would that be beneficial in heart failure treatment?

Heart failure remains to be a major public health problem. Its annual cost to the health care field is growing every year and currently stands at over 30 Billion US dollars. Despite remarkable advances in the treatment of coronary disease and the risk factors associated with it, the incidence and prevalence of heart failure has only increased. In spite of all this, there hasn’t been a major breakthrough in heart failure treatment for many decades. Of course targeting the gut to
treat the heart seems a bit far fetched at this point in time. However, the GI tract is a complex organ, perhaps even tantamount to having many organs in one. The experimental results presented here strengthen the case for a direct humoral link and interplay between the heart and the GI tract. Further understanding of this link and characterization of the signaling process involved is likely to be a major advance in heart failure treatment.
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APPENDICES
APPENDIX A

Abbreviations Used

ACE – Angiotensin converting enzyme
ANF - Atrial natriuretic factor
ANOVA - Analysis of variance
ANP – Atrial natriuretic peptide
AT1 – Angiotensin II receptor type 1A
BNP – B-type natriuretic peptide
cANF – c-Atrial natriuretic factor (peptide)
cGMP – Cyclic guanylyl mono phosphate
CNP – C-type natriuretic peptide
DMSO – Dimethyl sulfoxide
DNP – Deandropsis natriuretic peptide
FITC – Fluorescein-isothiocyanate
GC – guanylyl cyclase
GI – Gastrointestinal
GTP – Guanylyl tri phosphate
i.d. – Internal diameter
i.v. – Intravenous
i.P. – Intraperitoneal
KDa – Kilo Dalton
KHD – Kinase homology domain
KO – Knockout
LAD – Left anterior descending artery
LSD – Least square difference
LV – Left ventricle
MAP – Mitogen activated pathway
MI – Myocardial infarction
MKP – Mitogen activated kinase phosphatase
NPR-A – Natriuretic peptide receptor type –A
NPR-B - Natriuretic peptide receptor type –B
NPR-C - Natriuretic peptide receptor type –C
o.d. – Outside diameter
PBS – Phosphate buffered saline
PDE – Phosphodiesterase
PIC- Probe induction catheter
PKG – Protein kinase G
RFU – Relative fluorescence units
RIA – Radioimmunoassay
RV – Right ventricle
TNF – Tumor necrosis factor

VEGF – Vascular endothelium derived growth factor

WT – Wild type
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After six years of private practice in internal medicine, he joined the cardiac peptide lab of Dr. John R. Dietz as a PhD student in 2004 to pursue his interest in molecular workings of the heart.

He has won numerous awards including a WHO sponsored visiting scholarship to the University of Washington, honored as resident of the year and chief resident, elected to fellowship of the American College of Physicians, awarded the national science foundation’s integrative graduate research education and training scholarship and recently was given the new investigator travel award from the American Heart Association.

As part of this dissertation he has authored an original article and several abstracts and has made several presentations at regional and national meetings.