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**Brain Natriuretic Peptide Gene Expression and Secretion Following  
Stimulation with Pro-inflammatory Cytokines and Conditioned Medium from  
Allo-activated Mixed Lymphocyte Reactions**

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**Brain Natriuretic Peptide Gene Expression and Secretion  
Following Stimulation with Pro-inflammatory Cytokines and  
Conditioned Medium from Allo-activated Mixed Lymphocyte  
Reactions**

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**This thesis submitted on August 17<sup>th</sup>, 2004 to the Faculty of Graduate and  
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## ABSTRACT

Atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP) are cardiac-derived polypeptide hormones secreted by the heart. ANF and BNP play critical roles in maintaining fluid and electrolyte balance in both health and disease. Mechanisms of increasing ANF and BNP gene expression and secretion by mechanical or by vasoactive agonists have been thoroughly examined. However, recent experiments suggest that pro-inflammatory cytokines may also be important regulators of natriuretic peptide secretion. In this study, neonatal rat ventricular cardiocyte cultures were used to examine the effects of pro-inflammatory cytokines on natriuretic peptide gene expression and secretion. Incubation with IL-1 $\beta$  or TNF- $\alpha$  elicited a dose and time dependent significant increase in BNP mRNA and secretion, whereas ANF mRNA and secretion was not affected by treatment. The increase in BNP mRNA was inhibited by pre-incubation with a transcriptional inhibitor, actinomycin D. Moreover, the IL-1 $\beta$  and TNF- $\alpha$  mediated increase was not affected by pre-incubation with cycloheximide, suggesting that a translation-dependent increase in BNP mRNA stability was not involved in increasing BNP mRNA abundance. IL-1 $\beta$  and TNF- $\alpha$  rapidly increased phosphorylated p38 MAP kinase and MAP kinase activity. Inhibition of p38 MAP kinase with SB203580 completely abolished IL-1 $\beta$  and TNF- $\alpha$  stimulated BNP promoter activity, mRNA abundance and peptide secretion. NF- $\kappa$ B is another signaling molecule activated by IL-1 $\beta$  and TNF- $\alpha$ , however, results from experiments using a peptide inhibitor to NF- $\kappa$ B signaling

suggest that NF- $\kappa$ B is not important in transducing the IL-1 $\beta$  and TNF- $\alpha$ -mediated increase in BNP secretion. Other pro-inflammatory and immunoregulatory cytokines like IL-6, IL-2 and IFN- $\gamma$  did not alter either BNP or ANF secretion but co-treatment with both IL-1 $\beta$  and IFN- $\gamma$  maximally stimulated BNP secretion, suggesting cooperation between IL-1 $\beta$  and IFN- $\gamma$  signaling. Conditioned medium from an allogenic mixed lymphocyte reaction (MLR) was used in order to circumvent the omission of individual cytokines or their combination. Serum-free MLR conditioned medium (SF-MLR-CM) in 20, 50 and 100% proportions increased BNP but not ANF secretion with respect to the basal serum-free lymphocyte medium controls. Although, this increase was sensitive to p38 MAP kinase inhibition, it appears that IL-1 $\beta$  and TNF- $\alpha$  are not major active constituents in the SF-MLR-CM as neither ELISA nor soluble receptor antagonists to IL-1 $\beta$  or TNF- $\alpha$  revealed the presence of these pro-inflammatory cytokines. In summary, our novel findings reveal a unique discoordinated expression of BNP and ANF induced by pro-inflammatory cytokines. Importantly it offers an opportunity to better understand the differential regulation of these two cardiac-derived endocrine hormones that share receptors as well as biological properties.

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## ABBREVIATIONS

|               |  |
|---------------|--|
| ANF:          | Atrial natriuretic factor, atrial natriuretic peptide, A-type natriuretic factor |
| ANG-II:       | Angiotensin-II   |
| APC:          | Antigen presenting cell  |
| ATF:          | Activator of transcription   |
| ATP:          | Adenosine triphosphate   |
| AVP:          | Arginine vasopressin   |
| BNP:          | Brain natriuretic peptide, B-type natriuretic peptide                            |
| $\beta$ -gal: | $\beta$ -galactosidase   |
| $\beta$ -MHC: | $\beta$ -myosin heavy chain  |
| cGMP:         | Guanosine 3',5'-cyclic monophosphate   |
| CaM:          | Calmodulin   |
| CNP:          | C-type natriuretic peptide   |
| CT-1:         | Cardiotrophin-1  |
| DNA:          | Deoxyribonucleic acid  |
| DOCA:         | Deoxycorticosterone acetate  |
| EMSA:         | Electrophoretic mobility shift assays  |
| ERK:          | Extracellular signaling regulated kinase   |
| ET-1:         | Endothelin-1   |
| FAC:          | Focal adhesion complex   |
| FAK:          | Focal adhesion kinase  |

|                 |   |
|-----------------|---|
| FGF:            | Fibroblast growth factor  |
| GTP:            | Guanosine triphosphate  |
| HPLC:           | High performance liquid chromatography                              |
| IEG:            | Immediate early gene  |
| IGF1:           | Insulin growth factor 1   |
| IL-1 $\beta$ :  | Interleukin-1 $\beta$   |
| IL-2:           | Interleukin-2   |
| IL-4:           | Interleukin-4   |
| IL-6:           | Interleukin-6   |
| IMCD:           | Inner medullary collecting duct                                     |
| INF- $\gamma$ : | Interferon- $\gamma$  |
| iNOS:           | Inducible nitric oxide synthase                                     |
| ISO:            | Isoproterenol   |
| JAK-STAT:       | Janus kinase and signal transducers and activators of transcription |
| JNK:            | c-Jun N-terminal kinase   |
| KHD:            | Kinase homology domain  |
| LIF:            | Leukemia inhibitory factor  |
| LPS:            | Lipopolysaccharide  |
| MAP:            | Mitogen activated protein   |
| MEK:            | Mitogen activated protein kinase kinase                             |
| MHC:            | Major histocompatibility complex                                    |
| MLR:            | Mixed lymphocyte reaction   |
| mRNA:           | Messenger ribonucleic acid  |
| NEP:            | Neutral endopeptidase 24.11   |

|            |   |
|------------|---|
| NFAT:      | Nuclear factor of activated T-lymphocyte cells          |
| NF-κB:     | Nuclear factor-κB                                       |
| NO:        | Nitric oxide  |
| NPR:       | Natriuretic peptide receptor                            |
| NRVC:      | Neonatal rat ventricular cardiocyte                     |
| PDGFA:     | Platelet derived growth factor A                        |
| PCR:       | Polymerase chain reaction                               |
| PE:        | Phenylephrine   |
| PKC:       | Protein Kinase C  |
| PLC:       | Phospholipase C   |
| RAAS:      | Renin-angiotensin-aldosterone-system                    |
| RIA:       | Radioimmunoassay  |
| RT-PCR:    | Reverse transcriptase polymerase chain reaction         |
| SAPK:      | Stress-activated MAP kinase                             |
| SERCA:     | Sarcoplasmic reticulum Ca <sup>2+</sup> -ATPase         |
| SF-LM:     | Serum-free lymphocyte medium                            |
| SF-MLR-CM: | Serum-free mixed lymphocyte reaction conditioned medium |
| TCR:       | T-cell receptor   |
| TEF-1:     | Transcription enhancing factor-1                        |
| TGF-β:     | Transforming growth factor-β                            |
| TIMP:      | Tissue inhibitor of matrix metalloproteinase            |
| TNF-α:     | Tumour necrosis factor-α                                |
| YY1:       | Ying Yang 1   |

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## AUTHORIZATION

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Ma K.K., Banas K., and de Bold, A.J.; *Cis*-acting determinants of brain natriuretic peptide promoter activity. *Regulatory Peptides.* (In press, Apr 2004)

Ma K.K., and de Bold A.J.; Atrial natriuretic factor and family of natriuretic peptides. Chapter in *Encyclopedia of Endocrine Diseases.* Ed. Luciano Martini. *Academic Press.* 2004

## INTRODUCTION

The atrial natriuretic factor family of natriuretic peptides consists of three members: atrial natriuretic factor (ANF), brain natriuretic peptide or B-type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). These polypeptide hormones are structurally related and play central roles in maintaining fluid and electrolyte balance and cardiovascular homeostasis. ANF and BNP primarily function as potent natriuretic and diuretic hormones, but they also antagonize sympathetic activity and the renin-angiotensin-aldosterone-system (RAAS). CNP is slightly different in that it has vasodilatory and anti-proliferative effects on vascular smooth muscle (Clavell et al., 1993). Mice harboring mutations that disrupt the natriuretic peptides (John et al., 1995) or their receptors (Oliver et al., 1997) have higher than normal blood pressure and subsequently develop cardiovascular disease. By contrast, mice that overexpress natriuretic peptides (Steinhilper et al., 1990) or their receptors, develop hypotension (Oliver et al., 1998). These experiments highlight the importance of the natriuretic peptides in the maintenance of cardiovascular homeostasis in health and disease.

In mammals, under normal physiological conditions, the bulk of ANF and BNP is produced and secreted by the cardiac muscle cell (cardiocyte) in the atrium, whereas, CNP is predominately found in the vascular endothelium (Suga et al., 1992b) and in the central nervous system (Kojima et al., 1990). Acute or chronic hemodynamic overload or stimulation with vasoactive agonists such as endothelin-1 (ET-1), angiotensin II (ANG-II) and the  $\alpha_1$ -adrenergic agonist phenylephrine (PE)

results in increased expression of ANF and BNP in both the atria and ventricles. The increased production of the natriuretic peptides serves to reduce cardiac preload and afterload. The molecular mechanisms that upregulate the natriuretic peptides during hemodynamic overload and by vasoactive hypertrophic agonists have been thoroughly investigated. In fact, this upregulation of both ANF and BNP is so consistent that they have become useful biomarkers for altered myocardial structure and function seen in cardiac hypertrophy and congestive heart failure (Ruskoaho, 2003).

Basal plasma levels of both ANF and BNP remain elevated after heart transplantation despite normalization of cardiac function, hemodynamic parameters and RAAS activity (Masters et al., 1993). The precise reasons for this remain unclear. Furthermore, preceding and during an acute cardiac allograft rejection episode as determined by endomyocardial biopsy score, BNP plasma levels increase dramatically and decrease only after the treatment of rejection with anti-T-lymphocyte therapy (Masters et al., 1999). The increase of plasma BNP, without a concomitant increase in ANF is an exception to the general rule and it can be speculated that this selective increase in BNP is mediated in part by pro-inflammatory cytokines synthesized by lymphocyte interactions during cardiac allograft rejection. The purpose of this study was to examine the effects of pro-inflammatory cytokines on BNP gene expression and secretion and to elucidate some of the molecular mechanisms and signal transduction pathways used by pro-inflammatory cytokines to mediate selective increases in BNP expression. To our knowledge, the role of pro-inflammatory cytokines in the specific and selective upregulation of BNP synthesis and secretion has never been directly addressed.

## Historical Perspective of the Natriuretic Peptides

In 1948 Borst (Borst, 1948) hypothesized that small changes in blood volume would affect venous pressure and cardiac output (assuming no change in vascular compliance), which in turn alter the response of the kidney to water and salt excretion or reabsorption. This observation led the way in the search for the mechanism(s) responsible for cardiorenal regulation of blood volume. In 1956 Henry and Pearce reported that inflation of a balloon in the left atrium of dogs resulted in increased diuresis (Henry and Pearce, 1956). Atrial and arterial baroreceptors were known to exist, however, axotomy of the vagus failed to eliminate the natriuretic response to volume-loading (Henry and Pearce, 1956). At about the same time using electron microscopy, Kisch found that atrial cardiocytes had morphological features of secretory cells, an observation not seen for the ventricular cardiocytes of the guinea pig heart (Kisch, 1956). Jamieson and Palade later coined these membrane-bound granules, *specific atrial granules* (Jamieson and Palade, 1964). Bencosme and Berger noticed a similarity between the specific atrial granules and the juxtaglomerular granules of the kidney and postulated an endocrine function for the atrial cells (Bencosme and Berger, 1971). Additional experiments showed that the uptake of  $^3\text{H}$ -leucine resembled that of cells producing polypeptide hormones (de Bold and Bencosme, 1975). In 1981, de Bold *et al.* made the first demonstration that intravenous injection of atrial extracts into anesthetized rats caused an increase in natriuresis and diuresis by thirty and ten-fold respectively, and speculated of the existence of a natriuretic factor present in the cardiac atria (de Bold et al., 1981). Steps to purify the natriuretic factor from specific atrial granules were successful (de Bold, 1982) and soon afterwards the ANF amino acid sequence was obtained (Flynn

et al., 1983). The cloning of the ANF cDNA was accomplished in 1985 (Flynn et al., 1985; Argentin et al., 1985).

In summary, the initial discovery of ANF solidified the link between the secretory nature of the atrial cardiocytes and volume regulation by the kidneys. The additional members in the family of natriuretic peptides, BNP and CNP, were discovered in 1988 (Sudoh et al., 1988) and 1990 respectively (Sudoh et al., 1990). It is now firmly established that these three natriuretic peptides constitute an important system involved in regulating water and electrolyte balance, cell growth and proliferation in normal physiological and pathophysiological conditions.

## **Molecular Aspects of Brain Natriuretic Peptide**

BNP was originally isolated from the porcine brain (Sudoh et al., 1988) with similar peptides subsequently identified in human (Sudoh et al., 1989), rat (Kojima et al., 1989), mouse (Steinhelper, 1993) and dog (Seilhamer et al., 1989). The name brain natriuretic peptide is actually a misnomer, since in humans it is barely detectable in the brain and in fact, the highest BNP mRNA concentrations are found within the heart in the atrial cardiocytes (Ogawa et al., 1990; Ogawa et al., 1991; Mukoyama et al., 1991a). The BNP gene is a single copy gene located in close proximity to the ANF gene on chromosome 1 in humans and 4 in mouse (Steinhelper, 1993; Ogawa et al., 1995). BNP and ANF genes are structurally similar in that both are composed of 3 exons and 2 introns (Sudoh et al., 1989) (Figure 15). Transcription leads to a 900-bp long BNP mRNA which is unique among the natriuretic peptide family by having a 3'UTR that contains numerous AUUUA rich sequences (Kojima et al., 1989; Sudoh et al., 1989). These sequences are hypothesized to confer BNP mRNA transcript instability (Shaw and Kamen, 1986; Hanford et al., 1994).

## Biochemistry of BNP

In humans, the BNP biosynthetic pathway begins with cleavage of a 26 amino acid signal peptide to form a 108 amino acid BNP pro-hormone (BNP<sub>1-108</sub>) (Sudoh et al., 1989). BNP<sub>1-108</sub> is then cleaved by the pro-hormone endoprotease furin at an Arg-X-X-Arg motif (Sawada et al., 1997) to the inactive BNP<sub>1-76</sub> and active BNP<sub>77-108</sub> fragments (Figure 14). BNP<sub>77-108</sub> is co-stored along with pro-hormone ANF<sub>1-126</sub> in atrial secretory granules (Nakamura et al., 1991; Hasegawa et al., 1991; de Bold and Bruneau, 2000). Upon secretion, ANF<sub>1-126</sub> is cleaved into ANF<sub>99-126</sub> by the serine protease corin (Wu et al., 2002) and circulates with BNP<sub>77-108</sub> in plasma. Both ANF<sub>1-98</sub> and BNP<sub>1-76</sub> N-terminal fragments circulate in plasma, but their functions are currently unknown. The plasma half-life of BNP in humans is approximately 18 minutes (McGregor et al., 1990), about six times longer than that of ANF (Yandle et al., 1986). It is speculated that this may be related to a combination of a slightly reduced affinity for the natriuretic peptide clearance receptor, NPR-C (Holmes et al., 1993) and decreased rate of metabolism by the neutral endopeptidase 24.11 (NEP) (Kenny et al., 1993). The normal circulating BNP concentration in humans can range from 0.9 to 6 fmol/ml, which is about one-fifth of ANF plasma levels (Mukoyama et al., 1991a).

Of the natriuretic peptides, BNP is least conserved with only 59% of amino acids retained among species (Rosenzweig and Seidman, 1991). In sharp contrast, ANF and CNP peptide sequences are the better conserved between species, at 86% and 90% respectively (reviewed by (Flynn, 1996)). Furthermore, BNP may be found in plasma as fragments of different size depending on the species. In humans and pigs it is a 32 amino acid peptide (Sudoh et al., 1988; Sudoh et al., 1989) but in rats and

mice it is a 45 amino-acid peptide (Aburaya et al., 1989). The reasons for this stem from differences in exon II of BNP gene that cause the proline-arginine site where natriuretic peptide pro-hormone processing takes place, to be moved 13 amino acids closer to the C-terminus in the human and porcine genes than it is in the rat and mouse genes.

Regardless of species, the main structural feature conserved in all three natriuretic peptides is the 17-amino-acid ring connected by a disulfide bond (Figure 14). Activity of the ANF, BNP and CNP is completely abolished if the disulfide bond is reduced (Misono et al., 1984).

## Basal Regulation of BNP Gene Expression

In a normal physiological state, the cardiac-derived natriuretic peptides ANF and BNP are continuously released from the heart, primarily by the atrial cardiocytes. In the adult atria, ANF mRNA transcript levels are abundant at about 1-3% of total mRNA (Seidman et al., 1985) and atrial BNP mRNA levels are only slightly lower at about 50% of the atrial ANF mRNA levels. In the ventricles, ANF mRNA transcript levels are 1% of atrial ANF mRNA levels (Day et al., 1987) and ventricular BNP mRNA is much lower at about 1% of atrial BNP mRNA levels (Ogawa et al., 1991). Natriuretic peptide transcripts have also been detected in minute amounts in extra-cardiac tissues such as the aortic arch (Gardner et al., 1986), kidney (McKenzie et al., 1985; Greenwald et al., 1992; Golomb et al., 1993) and the hypothalamus (Gardner et al., 1987b), but the major site of production of ANF and BNP remains the cardiac atria under normal physiological conditions.

The marked differences in the basal levels of expression of ANF versus BNP genes in the heart allude to differences in the regulation of natriuretic peptides. This is not surprising since the 5'-promoter region of these two genes are quite different. The regulation of basal tissue-specific and inducible ANF mRNA expression by *cis*-acting elements on the ANF promoter have been the subject of intense study (Seidman et al., 1984; Seidman et al., 1988; LaPointe et al., 1988; Wu et al., 1989; Knowlton et al., 1991; Argentin et al., 1991; Rockman et al., 1991; McBride et al., 1993; Sprenkle et al., 1995; Knowlton et al., 1995). Unfortunately, knowledge of the regulatory elements involved in basal tissue-specific BNP gene expression is not as abundant.

It has been suggested that the structural organization of the proximal rat BNP (rBNP) promoter resembles more like an erythroid-cell specific promoter than a cardiac promoter (Grepin et al., 1994). For instance, *cis*-acting elements responsible for the regulation of hematopoietic cell expression such as a GATA box at -33-bp, a CCACC box that binds the EKLF-Krüppel family of zinc-finger proteins and an AP-1-like motif are present on the 5' proximal BNP promoter (McBride and Nemer, 2001). This is in contrast to the ANF promoter which is not arranged structurally in the same manner and contains characteristic *cis*-acting elements such as the CArG/serum response element that are commonly found in other cardiac gene promoters (Minty and Kedes, 1986). The proximal -114-bp rBNP promoter (relative to the transcription start site) contains two GATA zinc finger transcription factor DNA-binding elements (-90-bp/-81-bp) which confer the majority of basal and tissue-specific rat BNP expression (Grepin et al., 1994; Thuerauf et al., 1994). Another site involved in modulating basal rBNP expression is the M-CAT site (-109-bp to -102-bp), as mutation of this sequence within the proximal promoter significantly depressed basal gene expression (Thuerauf and Glembotski, 1997). With respect to the human BNP (hBNP) promoter, the majority of the regulatory domains governing tissue-specific hBNP gene expression also reside in the proximal promoter (Ogawa et al., 1995). These sequences include the GATA (-85-bp) (He et al., 2002) and the M-CAT element (-97-bp) (LaPointe et al., 1996). However, it appears that more distal regions, as far as -408-bp may also be important for maintaining basal and cardiac-specific hBNP expression (He et al., 2000; He et al., 2001).

## Inducible Regulation of BNP Gene Expression

In conditions of chronic hemodynamic overload and ventricular hypertrophy, the ventricular levels of BNP and ANF mRNA increase dramatically in humans (Takahashi et al., 1992). In the spontaneously hypertensive rat and other experimental models of heart failure (Ogawa et al., 1991; Dagnino et al., 1992) the increase in natriuretic peptide mRNA is mirrored by a significant rise in BNP and ANF plasma levels. In some instances, the fold-increase in BNP mRNA and secretion may exceed that of ANF, further suggesting that there are differences in the regulation of ANF and BNP genes even by the same stimuli. The bulk of the knowledge concerning the mechanisms involved in the inducible regulation of natriuretic peptide gene expression and secretion have come from *in vivo* or *in vitro* experiments examining the effects of mechanical and/or vasoactive hypertrophic agonists. Therefore, at present, very little information about the mechanisms involved in the regulation of natriuretic peptide transcription by pro-inflammatory cytokines is available. However, it may be reasonable to assume that at least some of the mechanisms and pathways that are used by mechanical and vasoactive hypertrophic agonists are also used by some pro-inflammatory cytokines to increase natriuretic peptide gene expression and secretion.

Chronic mechanical stretching (Izumo et al., 1988) or stimulation of cultured neonatal rat ventricular cardiocytes with ANG-II (Baker and Aceto, 1990; Sadoshima and Izumo, 1993), ET-1 (Shubeita et al., 1990; Ito et al., 1991) or PE (Knowlton et al., 1991) leads to the re-expression of a gene program seen during cardiac ventricular development. This program is characterized by the rapid expression of genes that do not require *de novo* protein synthesis, the so-called "immediate early genes" (IEGs)

such as *c-myc*, *c-fos* and *c-jun* (Izumo et al., 1988; Komuro et al., 1988a; Komuro et al., 1988b; Shubeita et al., 1990; Komuro et al., 1990; Neyses and Vetter, 1992; Bruneau and de Bold, 1994). These IEGs may be responsible in-part for activating many “late activating genes” encoding multiple contractile proteins such as ventricular myosin light chain-2 (MLC-2v) (Lee et al., 1988),  $\alpha$ -skeletal actin (Schwartz et al., 1986),  $\beta$ -myosin heavy chain (Izumo et al., 1987) and non-contractile secretory proteins such as ANF and BNP (Knowlton et al., 1991) during ventricular hypertrophy.

### **Mechanical regulation of BNP transcription**

*In vitro* application of direct mechanical strain to isolated cardiocytes grown on stretchable substrata demonstrated that cyclic strain induced a two-fold increase in the activity of a -1595-bp hBNP promoter and BNP mRNA expression. The increase in gene expression was inhibited by the transcriptional inhibitor actinomycin D, suggesting that transcriptional mechanisms were responsible for this upregulation (Liang et al., 1997).

It is now known that several *cis*-acting regulatory elements on the 5'-flanking BNP promoter are important for BNP gene induction following cardiocyte strain. The GATA elements in the 5'-proximal region of the promoter may play a major role in transducing mechanical stress stimulus into increased BNP gene expression. There are 6 members of the GATA family of zinc finger transcription factors. GATA-4, -5 and -6 are expressed in the heart. GATA-4 is believed to regulate the expression of many cardiac specific genes including the cardiac  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (Nicholas and Philipson, 1999), cardiac  $\alpha$ -myosin heavy chain ( $\alpha$ -MHC) (Molkentin et al., 1994) and ANF (Grepin et al., 1994). Mechanical stretch transiently increases GATA-4

binding to GATA DNA consensus elements and GATA mRNA transcript levels in cell culture (Pikkarainen et al., 2003b). Using site-directed mutagenesis and various truncation mutants, Pikkarainen *et al.* proposed that stretch-responsiveness requires at least –520-bp of the rBNP promoter (Pikkarainen et al., 2003b) as mutation of the proximal GATA elements at –90-bp and –81-bp only decreased stretch-induced rBNP promoter activation by 40%. Experiments using direct gene transfer of the rBNP promoter into the myocardium of an adult rat with bilateral nephrectomy to increase pressure and volume overload show that these GATA sequences on the proximal promoter are necessary for transducing hemodynamic stress stimulus to increased BNP transcription *in vivo* (Marttila et al., 2001). However, to elicit the maximum stretch-mediated responses, additional proximal and/or distal consensus elements and the GATA proteins may require the binding of additional co-factors to the BNP promoter that are not normally associated with a stretch-mediated response. In support of this idea, mutation of the proximal GATA elements and the Nkx2.5-like element (NKE) at –387-bp virtually abolished the stretch-mediated increase in rBNP promoter activity (Pikkarainen et al., 2003b). The cooperation between GATA-4 and Nkx2.5 is not unprecedented, as this has also been observed for the basal expression of other cardiac-specific genes (Durocher et al., 1997).

The activator protein-1 (AP-1) transcription factor complex is another factor that may cooperate with GATA to directly regulate the stretch-induced increase in BNP transcription. AP-1 is the binding site for the products of two immediate-early genes, *c-fos* and *c-jun*. AP-1 binding activity is increased in animals with aortic coarctation-induced pressure overload (Herzig et al., 1997) and application of direct left ventricular wall stress to isolated perfused hearts (Hautala et al., 2002). *C-fos* and

*c-jun* have been shown to regulate the expression of many genes associated with cardiac hypertrophy including ANG-II receptor 1a (AT<sub>1a</sub>R) (Herzig et al., 1997) and ANF (Kovacic-Milivojevic et al., 1996). Similar to the rANF promoter, the rBNP promoter also contains numerous AP-1 binding sites (Thuerauf et al., 1994). Although mutation of an AP-1-like site (–100-bp) in the context of a truncated –114-bp rBNP promoter inhibited basal activity of rBNP promoter, it did not have an effect on the stretch-mediated response of the promoter (Marttila et al., 2001). This finding was corroborated by a recent study showing that the AP-1 transcription factor may not contribute significantly to mechanical stress-induced BNP promoter activity (Pikkarainen et al., 2003b). It is important to keep in mind that a portion of strain-activated BNP gene expression *in vivo* may be due to the stretch-activated paracrine release of vasoactive factors such as ET-1 and ANG-II from the endothelial and fibroblast cells respectively (Liang and Gardner, 1998).

Additional *cis*-acting elements that may be partly responsible for the induction of BNP by mechanical stress are the shear stress responsive elements (SSREs) located in the proximal hBNP promoter at positions –153-bp, –633-bp, and –644-bp (Liang and Gardner, 1999). Interestingly, Liang *et al.* demonstrated that in response to cyclic mechanical stretch there is an increase in NF- $\kappa$ B binding to the SSRE sequences as determined by electrophoretic mobility shift assay (EMSA). NF- $\kappa$ B is a transcription factor that is commonly activated by pro-inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  in immune cells to upregulate cytokine gene expression (Saklatvala et al., 1999). Single or multiple site-directed mutations of the SSRE elements or co-transfection with a constitutive suppressor of NF- $\kappa$ B activity caused a maximum 40% decrease in strain-activated hBNP promoter activity. These effects were mimicked by the use of the p38

MAP kinase inhibitor, SB203580, suggesting that a component of strain-activated BNP transcription may also depend on signaling by p38 MAP kinase. However, these specific SSREs are not present on the 5'-flanking region of the rBNP, even though a moderate 65% homology exists between rat and human genes (LaPointe et al., 1996) and strongly suggest that perhaps other uncharacterized *cis*-acting stress sensitive elements may be present on the rBNP gene highlighting some of the species differences in the regulation of the BNP gene.

Several recent studies have documented the importance of outside-in signaling by extracellular matrix (ECM) proteins in the heart during cardiac pathologies such as cardiac hypertrophy (Ross et al., 1998; Ogawa et al., 2000). In particular, some have suggested that signaling via the ECM protein cell surface receptor, integrin, may play a role in translating mechanical stress to changes in gene expression and the induction of ANF and BNP (Aikawa et al., 2002). Direct evidence for the importance of the interaction between the integrins and activation of the hBNP promoter by mechanical strain was provided by Liang *et al.* (Liang et al., 2000a). They showed that disruption of the fibronectin/integrin interaction results in decreased strain-dependent BNP promoter activity. However, the sequence(s) of the 5'-flanking region of the -1.8-kbp hBNP promoter targeted by integrin signaling were not determined (Liang et al., 2000a). A recent report by Ogawa *et al.* demonstrated that basal hBNP gene transcription is upregulated in non-stretched cardiocytes by fibronectin signaling. Mutation of the neuron-restrictive silencer element (NRSE) in the 5'-flanking region between -552 and -522-bp of the hBNP promoter or transfection of a dominant negative neuron-restrictive silencer factor (NRSF) increased basal BNP promoter activity significantly only when the cardiocytes were plated on fibronectin coated

dishes (Ogawa et al., 2002; Liang et al., 2003). It is not known if a NRSE-dependent mechanism is responsible for myocardial stretch-induced BNP transcription.

### **Vasoactive agonist-induced BNP transcription**

Vasoactive neurohumors such as ET-1, ANG-II and certain adrenergic agonists play major roles in the development of cardiac hypertrophy and have been shown to increase BNP mRNA transcription and secretion. ET-1, a G<sub>q</sub> protein-coupled receptor hypertrophic agonist induces a significant increase in BNP (and ANF) gene expression and secretion (Shubeita et al., 1990; Ito et al., 1991), however, the precise mechanism(s) by which BNP transcription is increased by ET-1 still remains unclear. In cardiocytes, the major ET-1 receptor is the ET<sub>A</sub> subtype and it is widely accepted that the ET<sub>A</sub> receptor activates the phospholipase C (PLC), protein kinase C (PKC) and MAP kinase cascades (Kovacic et al., 1998). The ET<sub>B</sub> receptor subtype is found primarily on endothelial cells and the exact signal transduction pathways downstream of the receptor are unclear, but it has been shown to activate protein kinase B and nitric oxide (NO) generation (Liu et al., 2003). Recent experimental evidence suggest that for maximum ET-1-stimulated activation of the rBNP promoter, an intact E-twenty six (ETS) binding sequence (EBS) at position –498-bp is required (Pikkarainen et al., 2003a). In the context of a truncated –534-bp rBNP promoter, mutation experiments demonstrated that the EBS responds exclusively to ET-1 signaling and is not involved in mechanical stretch- or ANG-II-mediated increases in BNP promoter activity. Furthermore, it appears that other major *cis*-acting elements such as a distal AP-1 (–373-bp) site and the proximal GATAs (–90/–81-bp) are not major determinants of ET-1-mediated increases in rBNP transcription. This is consistent with other reports that showed that although ET-1

increased GATA-4 DNA-binding (Kerkela et al., 2002), the mutation of the GATA sites does not abrogate ET-1-stimulated rBNP promoter activity (Pikkarainen et al., 2002). Unlike the rBNP promoter, the regulation of the hBNP promoter by ET-1 appears to rely on both distal and proximal promoter elements. Truncation and site-directed mutagenesis experiments demonstrated that disruption of the GATA element (-85-bp) within a -408-bp hBNP promoter, only decreased total ET-1-stimulated increase in promoter activity by about 50%. Thus, the region between the -1.8-kbp and -408-bp of the hBNP promoter contains potential ET-1 responsive *cis*-acting elements that have yet to be characterized (He and LaPointe, 2001).

ANG-II, another G<sub>q</sub> protein-coupled receptor hypertrophic agonist, also increases BNP mRNA expression and protein secretion (Wiese et al., 2000; Suo et al., 2002), but very little is known about ANG-II-mediated activation of the BNP promoter. It has been reported that ANG-II (similar to ET-1) stimulates GATA-4 binding in the heart, but GATA (-90/-81-bp) binding site mutation analysis has determined that these elements are not critical in mediating ANG-II-induced increases in rBNP activity (Pikkarainen et al., 2002).

$\alpha_1$ -adrenergic stimulation dramatically increases the expression of BNP mRNA and secretion in cultured isolated cardiocytes (Hanford et al., 1994). Interestingly, it has been suggested that the hypertrophic agonist, PE, stimulates BNP in a manner similar to that of an IEG with transcriptional induction being very rapid and not requiring *de novo* protein synthesis (Hanford et al., 1994). PE has been shown to increase rBNP transcription by activating the proximal GATA elements (-90/-81-bp). Single mutation of either GATA site reduced maximum PE-induced

BNP transcription by 50% while removal of both GATA elements resulted in the complete inhibition of PE-inducible promoter activity (Thuerauf et al., 1994). An additional *cis*-acting element that may confer PE-induced increases in promoter activity is the muscle-CATTCCT (M-CAT) consensus element. M-CATs and the transcription factor TEF-1 are important in regulating some other cardiac-specific genes such as  $\beta$ -myosin heavy chain (Kariya et al., 1994). Loss of the MCAT element (between -109-bp and -102-bp) completely abolishes PE-inducible rBNP transcription (Thuerauf and Glembotski, 1997). PE also increases the activity of the hBNP promoter. However, PE-sensitive elements on the hBNP promoter have not been fully characterized. It is known that the proximal GATA element (-85-bp), although critical for maintaining basal expression of BNP, does not appear to play a role in PE-inducibility of the hBNP promoter (He et al., 2002). Similarly, the M-CAT sequence (-97-bp) does not appear to play a major role in PE-inducible hBNP transcription (He et al., 2000).

$\beta$ -adrenergic agonists have also been shown to upregulate BNP transcription. Isoproterenol (ISO) induced hBNP activity is believed to be conferred by both GATA (He et al., 2002) and M-CAT consensus elements (He et al., 2000). However, it is not known if there exists an interaction between GATA and M-CAT elements on the hBNP promoter as elimination of either GATA (-85-bp) or M-CAT (-97-bp) elements significantly reduces (by 60% and 96%, respectively) ISO-induced increases in hBNP promoter activity (He et al., 2000; He et al., 2002).

### **Other agonists that induce BNP transcription**

A mediator of inflammation, lipopolysaccharide (LPS) can also regulate the expression of the BNP gene. The release of LPS from Gram-negative bacteria results in an acute inflammatory response that can cause an increase in myocardial depression accompanied by profound changes in cardiac gene expression (Hung and Lew, 1993). Recent experiments have reported that LPS significantly upregulates BNP mRNA in rat heart. Transfection experiments with a –1000-bp rBNP promoter have shown that LPS targets the GATA elements located in the proximal rBNP promoter to increase activity by 2.1-fold (Tomaru et al., 2002). However, when the promoter is truncated to –112-bp, the LPS-induced activity is increased to 4.1-fold, suggesting the existence of important uncharacterized negative regulatory elements between –112-bp and –1000-bp. The precise signaling mechanisms responsible for upregulating the rBNP promoter activity by LPS was not examined in this study, but what is known about the LPS receptor is that it belongs to the toll-like receptor family and can signal through transcription factors such as NF- $\kappa$ B (Palsson-McDermott and O'Neill, 2004).

Thyroid hormones can regulate growth and gene expression of many cardiocyte genes including ANF (Gardner et al., 1987a), sarcoplasmic endoplasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA) (Hartong et al., 1994) and  $\alpha$ -myosin heavy chain (Kinugawa et al., 2001). It has been established for sometime that T<sub>3</sub> and T<sub>4</sub> can stimulate BNP secretion *in vivo* and *in vitro* (Kohno et al., 1993b). It has been recently found that T<sub>3</sub> stimulated a 3-fold increase in activity of a –1595-bp hBNP promoter by binding to a thyroid hormone response element (TRE) around position –

1000-bp (Liang et al., 2003). Mutation of the TRE completely abolishes T<sub>3</sub>-mediated increases in BNP promoter activity but does not affect basal promoter activity.

Increased levels of many pro-inflammatory cytokines have been found in patients with myocardial infarction, congestive heart failure (Levine et al., 1990) and following heart transplantation (Duquesnoy and Demetris, 1995). Recent studies suggest a mechanistic link between pro-inflammatory cytokines and the stimulation of BNP gene expression. During acute cardiac allograft rejection, BNP plasma levels are significantly increased (Masters et al., 1999). *In vitro* studies have demonstrated that conditioned medium from allo-activated T-lymphocyte cell cultures, a model of transplantation immunity, selectively upregulates BNP but not ANF expression in cultured rat neonatal ventricular cardiocytes (Ma et al., 2004). Furthermore, Harada *et al.* have shown that the pro-inflammatory cytokine IL-1 $\beta$  can increase BNP secretion (Harada et al., 1999) and Ma *et al.* have shown that both IL-1 $\beta$  and TNF- $\alpha$  can not only increase BNP secretion, but mRNA and promoter activity levels are also increased by these cytokines (Ma et al., 2004). The precise 5' *cis*-acting regulatory regions on the BNP promoter responsible for this phenomenon have not been identified, but it has been demonstrated that IL-1 $\beta$  can target the M-CAT element (-97-bp) in the proximal hBNP promoter (He and LaPointe, 1999). Although ablation of the M-CAT element reduces IL-1 $\beta$ -mediated BNP expression, the large proportion of activity still remaining implies that other uncharacterized factors are involved in mediating the increased BNP transcription.

## **Intracellular Signaling Pathways used to Increase BNP Gene Expression**

The secondary signaling mechanisms used by mechanical or vasoactive hypertrophic agonists to upregulate BNP gene expression and secretion have been intensely explored. Numerous signaling pathways have been proposed including  $\text{Ca}^{2+}$  and the MAP kinase family. Recent evidence that pathways activated by certain growth factors and numerous pro-inflammatory cytokines also mediate the upregulation of natriuretic peptide gene expression, however, at present these pathways are not well characterized.

### **The role of calcium**

It is established that increased mechanical stimulus causes the cardiocyte to increase natriuretic peptide gene expression and secretion, but the precise receptor and signaling pathways by which the force input is translated into biochemical stimulus has not been completely elucidated. A mechanosensitive nonselective cation channel that is sensitive to inhibition by the trivalent lanthanide, gadolinium ( $\text{Gd}^{3+}$ ) was described by Yang and Sachs in *Xenopus* oocytes (Yang and Sachs, 1989). These stretch activated (SA) channels have been localized on chick ventricular cardiocytes and it was hypothesized that they may be involved in the cardiocyte mechanotransduction process (Sigurdson et al., 1992; Kim and Fu, 1993; Kim, 1993). SA channels allow the movement  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{2+}$  ions (Ruknudin et al., 1993). Therefore, the role of  $\text{Ca}^{2+}$  as a secondary messenger mediating stretch-induced increase in ANF and BNP secretion was proposed. In support of this theory,  $\text{Gd}^{3+}$  has been shown to inhibit the stretch stimulated expression of ANF and BNP mRNA expression (Laine et al., 1994; Laine et al., 1996). Conversely, it has been shown also that blockade of this SA channel with  $\text{Gd}^{3+}$  was ineffective in inhibiting expression of

stretch-stimulated immediate early genes (Sadoshima et al., 1992), a key step in activation of late activating genes such as ANF and BNP. Furthermore, others have demonstrated that diminishing extracellular  $\text{Ca}^{2+}$  may actually increase stretch-mediated increase in natriuretic peptide secretion suggesting that  $\text{Ca}^{2+}$  may actually be a negative modulator of natriuretic peptide release in the acute setting (de Bold and de Bold, 1989; Kuroski-de Bold and de Bold, 1991; Deng and Lang, 1992). Nonetheless,  $\text{Ca}^{2+}$  may be important in mediating long-term changes in cardiac structure, function and gene expression. Inhibition of the  $\text{Ca}^{2+}$ -binding protein, calmodulin (CaM) and CaM kinase (CaMK) (Liang et al., 1997; Kudoh et al., 2003) attenuate mechanical strain-activated BNP transcription, raising the possibility that  $\text{Ca}^{2+}$ -CaM and calcineurin (a  $\text{Ca}^{2+}$ -CaM-dependent phosphatase) pathways may be a downstream targets of the putative mechanosensitive cation channel. Calcineurin, activates nuclear factor of activated T-lymphocyte cells (NFAT) and is believed to be a critical element in the development of cardiac hypertrophy and the activation of ANF and BNP gene expression *in vivo* and *in vitro* (Molkentin et al., 1998; Molkentin, 2000; Fiedler et al., 2002).

### **The MAP kinase superfamily**

As mentioned in the previous section, another potential candidate in the mechanotransduction process may be the integrins (Aikawa et al., 2002). Integrins have been shown to be involved in stretch-stimulated changes in BNP gene expression in cell culture (Liang et al., 2000a). Ventricular overload in the intact animal results in increased integrin association with at least two non-receptor protein tyrosine kinases, focal adhesion kinase (FAK) and c-Src (Kuppuswamy et al., 1997). FAK and c-Src are found at the focal adhesion complex (FAC) where signaling

molecules are brought in close proximity to each other. FAK activates c-Src (Parsons and Parsons, 1997), which ultimately lead to the stimulation of extracellular signal-related kinase mitogen activated protein kinase (p44/42 ERK MAP kinase) via the SH2 domain proteins, Grb2 and Sos, and low molecular weight GTP-binding proteins *Ras* and *Raf-1* (Force and Bonventre, 1998).

The MAP kinases can be divided in three major subfamilies, ERK, c-Jun N-terminal kinase (JNK) and p38 MAP kinases. All are proline directed serine/threonine kinases that phosphorylate numerous cytoplasmic and nuclear substrates. The activation of ERK, JNK and p38 follows a well-known generic sequential pathway beginning with activation of MAPKKK (MAP kinase kinase kinase) followed by a MAPKK (MAP kinase kinase) ending with the terminal MAP kinase. In the case of ERK pathway, *Ras* stimulates the MAPKKK, *Raf-1*, which activates MAPKK (also known as MEK1) to stimulate MAP kinase.

Vasoactive hypertrophic agonists such as ANG-II, ET-1 and PE bind heptahelical heterotrimeric GTP-binding protein receptors of the  $G_{\alpha q}$  subclass and robustly activate the ERK MAPK pathway (Bogoyevitch et al., 1993; Clerk et al., 1994; Thorburn et al., 1994; Yue et al., 2000). The ERK MAP kinase is believed to be important in upregulating natriuretic peptide gene expression and promoter activity since it has been shown that dominant negative forms of *Ras* or *Raf* inhibit PE-induced ERK MAP kinase and ANF promoter activity (Thorburn et al., 1993; Thorburn, 1994; Thorburn et al., 1994). Inhibition of MEK1 with PD98059 significantly decreases ET-1-induced BNP promoter activity (Liang et al., 2000b). Transfection of a constitutively active or dominant negative MEK1 activates or

inhibits respectively, ANF promoter activity (Gillespie-Brown et al., 1995), further supporting the role of ERK MAP kinase in the activation of the natriuretic peptides.

The JNK and p38 MAP kinases are known collectively as the stress-activated MAP kinases (SAPK) since they are stimulated by not only mitogenic growth factors but also in response to UV light, osmotic and shear stress and pro-inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  (Clerk et al., 1999; Bogoyevitch, 2000). In aortic banded MEKK1<sup>-/-</sup> (a MAPKKK) mutant mice, Sadoshima *et al.* proposed that JNK activation induces genes like transforming growth factor- $\beta$  (TGF- $\beta$ ) and TNF- $\alpha$  that are cardioprotective against the apoptosis and inflammation seen during chronic hemodynamic overload (Sadoshima et al., 2002). Furthermore, it appears that the JNK pathway may also be important in activation of the natriuretic peptides, since an adenoviral gene transfer of dominant-negative MKK4 (a MAPKK) suppressed the pressure-overload increase in ANF gene expression (Choukroun et al., 1999). In a similar manner, p38 MAP kinase may also be important in hypertrophy and upregulation of natriuretic peptides during pressure-overload (Thuerlauf et al., 1998; Wang et al., 1998; Nemoto et al., 1998) and by ET-1 and PE (Clerk et al., 1998). To increase gene expression during hypertrophy, p38 MAP kinase has been known to activate many important cardiac transcription factors such as transcription enhancing factor-1 (TEF-1) and NFAT3 (He and LaPointe, 1999; Yang et al., 2002).

### **The Janus kinase and signal transducers and activators of transcription (JAK-STAT)**

Another common signaling pathway employed by growth factors and cytokines is the Janus kinase (JAK) and signal transducers and activators of

transcription (STAT) pathway to increase natriuretic peptide gene expression in the heart. JAKs are cytoplasmic tyrosine kinases that phosphorylate STATs. The activated STATs translocate to the nucleus where they bind to DNA consensus elements in the promoter of target genes. Recently it has been shown that cardiotrophin (CT-1), a cardiac-specific cytokine and leukemia inhibitory factor (LIF) can increase BNP (Kuwahara et al., 1998; Hamanaka et al., 2000) and ANF mRNA expression using the JAK-STAT pathway (Wollert and Chien, 1997; Kunisada et al., 1998).

In summary, mechanical, vasoactive hypertrophic growth agonists and cytokines activate multiple secondary signaling pathways in cardiocytes to effect changes in gene expression. It appears that many of these pathways can ultimately activate members of the MAP kinase superfamily, in particular the p44/42 ERK MAP kinase. In contrast to mechanical and vasoactive agonists, it appears that pro-inflammatory cytokines primarily activate the JNK and p38 SAPK subfamily of the MAP kinases. Lastly, in the heart, it appears that numerous growth factors and cytokines also activate the JAK-STAT pathway to mediate changes in cardiac gene expression.

## **Guanylate Cyclase Natriuretic Peptide Receptors and Natriuretic Peptide Metabolism**

Three natriuretic peptide receptors, NPR-A, NPR-B and NPR-C have been purified (Kuno et al., 1986; Paul et al., 1987; Schenk et al., 1987) and cloned (Chinkers et al., 1989) (Fuller et al., 1988; Chang et al., 1989). The particulate guanylyl cyclase natriuretic peptide receptors, NPR-A and NPR-B, are present on many target tissues most notably the glomerulus and the renal inner medullary collecting duct (IMCD) of the kidneys (Mantyh et al., 1985), adrenal cortex zona glomerulosa (Bianchi et al., 1985; Mantyh et al., 1986), and vasculature (Hirata et al., 1984; Hirata et al., 1985) but these receptors are also found in the central nervous system (Quirion et al., 1986; Quirion et al., 1988; Quirion, 1989).

NPR-A and NPR-B are about 130 kDa polypeptides with an N-terminal extracellular domain, a single transmembrane region and a C-terminal cytoplasmic tail (reviewed by (Suga et al., 1992a)) (Figure 16). NPR-A and NPR-B share only a 44% amino acid sequence homology in the N-terminal ligand-binding domain (Schulz et al., 1989; Chinkers et al., 1989) which may be the reason behind the selectivity of these receptors for the natriuretic peptides. While NPR-A can bind both ANF and BNP, a slight preference is given to ANF, and although NPR-B primarily binds CNP, it may also bind BNP, but with much lower affinity (Chinkers and Garbers, 1991; Koller et al., 1991). On the other hand, the intracellular regions of NPR-A and NPR-B are 78% identical and both contain a kinase homology domain (KHD) and a C-terminal guanylyl cyclase catalytic domain. Binding of a ligand results in ATP hydrolysis which allosterically removes the inhibitory effect of the KHD on the guanylyl cyclase catalytic domain (Kurose et al., 1987) permitting the conversion of

guanosine triphosphate (GTP) into guanosine 3',5'-cyclic monophosphate (cGMP) (Lowe et al., 1989). cGMP is the secondary messenger that mediates most of the cardiovascular and renal effects of ANF and BNP.

NPR-C binds to all three natriuretic peptides and its primary role is that of a clearance receptor for the natriuretic peptides. This receptor is widely distributed in many tissues including the renal cortex, vascular and endothelial cells (Leitman and Murad, 1986; Nussenzveig et al., 1990; Suga et al., 1992a). The ligand binding domain of NPR-C is >75% identical with NPR-A and NPR-B (Porter et al., 1990) but NPR-C has a short C-terminal cytoplasmic tail and does not have either a KHD or guanylate cyclase catalytic domain (Fuller et al., 1988). Upon binding the natriuretic peptides, the entire complex is internalized via endocytosis, followed by lysosomal degradation and receptor recycling (Maack et al., 1987). Recent evidence suggests that this receptor can also participate in intracellular signaling via the inhibition of adenylate cyclase (Anand-Srivastava and Cantin, 1986) by  $G_i$  protein activation (Murthy et al., 2000), however, whether the activation of the  $G_i$  protein plays a role in additional signal transduction by ANF, BNP or CNP is not currently known.

Another mechanism for natriuretic peptide inactivation is by the neutral endopeptidase 24.11 (NEP) which enzymatically opens the 17-member ring structure of the natriuretic peptides (Erdos and Skidgel, 1989). NEP is predominantly bound to the plasma membrane by a hydrophobic N-terminal membrane-spanning domain and is present in the highest concentrations in renal tubular cells (Erdos and Skidgel, 1989) and to a lesser degree in cells of the vasculature (Tamburini et al., 1989) but NEP can also be found in a soluble form in plasma (Yandle et al., 1989). Recent

evidence suggests that the efficiency of NEP for ANF and BNP may be slightly different (Walther et al., 2004).

## **Physiological Actions of Brain Natriuretic Peptide**

Compared with ANF, relatively little research has been done on BNP with regards to its systemic actions. It has been shown that under normal conditions, BNP possesses natriuretic, diuretic and vasorelaxant properties that are similar to those found for ANF (McGregor et al., 1990; Kita et al., 1991; Richards et al., 1993; Hunt et al., 1996). BNP and ANF bind with high affinity to the same NPR-A receptor and it is possible in certain circumstances, to extrapolate the large amount of the data collected from the study of the actions of ANF to the actions of BNP, noting however, that some differences between BNP and ANF have been recently revealed with genetic approaches (Tamura et al., 2000; Chusho et al., 2000).

### **Actions on the renal system**

The natriuretic and diuretic actions of the natriuretic peptides result from a combination of the effects on renal hemodynamics, tubular sodium reabsorption and renin secretion. The NPR-A receptor is found on many sites in the kidney (Gunning et al., 1990). ANF and BNP increase single nephron glomerular filtration rate (GFR) by simultaneously stimulating afferent arteriole dilation and efferent arteriole constriction to raise glomerular capillary pressure (Marin-Grez et al., 1986). Furthermore, ANF and BNP-mediated increases in cGMP accumulation directly cause the relaxation of the glomerular mesangial cells, thereby increasing the available surface area for filtration. Another mechanism proposed by Kohno *et al.* (Kohno et al., 1993a) is that BNP may inhibit AVP-induced ET-1 mediated contraction of the glomerular mesangial cell (Bakris et al., 1991; Sakamoto et al., 1992).

At the level of the inner medullary collecting duct (IMCD) epithelial cells, ANF and BNP cause an inhibition of amiloride-sensitive  $\text{Na}^+$  reabsorption (Gunning et al., 1990; Sonnenberg et al., 1990; Sonnenberg, 1990) and promote  $\text{Na}^+$  influx into the tubule by stimulating the basolateral furosemide-sensitive  $\text{Na}^+/\text{K}^+/\text{2Cl}^-$  cotransporter (Maack, 1996). The effects of BNP on the IMCD are likely due to the increase in cGMP, since NPR-A knockout mice lose the natriuretic response (Lopez et al., 1995; Dubois et al., 2000). Furthermore, the effects of the membrane-permeable cGMP analogue, 8-bromo-cGMP mimic the natriuretic peptide-induced increase in  $\text{Na}^+$  uptake in cultured IMCD cells (Zeidel et al., 1987; Zeidel et al., 1988).

Infusion of ANF (Burnett, Jr. et al., 1984) and BNP (Jensen et al., 1998) markedly lowers renin secretion and circulating concentrations. These effects may be due to both indirect and direct actions of BNP on the kidney. BNP-induced natriuresis will decrease solute delivery to the macula densa cells which will decrease renin production (Akabane et al., 1991). In primary cultures of juxtaglomerular cells, ANF has been shown to inhibit basal renin release, an effect dependent on cGMP (Kurtz et al., 1986). Therefore, it is likely that the inhibition of renin by BNP may be due to direct effects involving stimulation of the particulate guanylate cyclase receptor and indirect effects due to renal autoregulation.

#### **Actions on the cardiovascular system**

The dose-dependent decrease in blood pressure caused by BNP results from the ability to reduce cardiac output (van der et al., 2003) and peripheral vascular resistance (Lainchbury et al., 1997). BNP-induced lowering of cardiac output is

initially mediated in-part by the inhibitory effects of this peptide on sympathetic nerve activity (Brunner-La Rocca et al., 2001). In the long-term, decreases in cardiac output can be attributed to decreases in cardiac preload due to dilation of capacitance veins and the shift of fluid from the intravascular to the extravascular compartment (Nakamura et al., 1998).

### **Actions on the central nervous system**

The effects of the natriuretic peptides on the central nervous system (CNS) generally reinforce their systemic effects on electrolyte balance and fluid volume and vascular tone (Samson, 1988). Although the plasma natriuretic peptides cannot cross the blood-brain barrier, they do reach sites immediately outside such as the circumventricular organs (e.g. *organum subfornicale*) (Saavedra et al., 1986; Saavedra, 1986) and the hypothalamic median eminence (Inagami et al., 1989; Levin, 1989) which are believed to be involved in regulation of cardiovascular tone and fluid homeostasis respectively. Furthermore, ANF immunoreactive neurons have been detected in the brain revealing the existence of mechanisms that control the local production and subsequent action of the natriuretic peptides in the brain (Saper et al., 1985)(reviewed by (Gutkowska et al., 1997)). Intracerebroventricular (i.c.v.) injection of ANF attenuates salt and water appetite in rats (Itoh et al., 1986; Blackburn et al., 1995). I.c.v. administration of BNP and CNP decreases arginine vasopressin (AVP) release (Yamada et al., 1988; Makino et al., 1992; Shirakami et al., 1993). Based on the larger number and wider distribution of NPR-B than NPR-A receptors in the CNS, it is generally accepted that CNP is the primary natriuretic peptide that participates in sodium and water balance in the CNS (Imura et al., 1992; Antunes-Rodrigues et al., 2004).

### **Actions on other neuroendocrine systems**

The NPR-A receptor is present on the zona glomerulosa cells of the adrenal gland (De Lean et al., 1984). BNP activation of the NPR-A receptor causes an increase in cGMP levels which inhibit ANG-II- and adrenocorticotrophic hormone (ACTH)-stimulated aldosterone production from adrenocortical cells (Hashiguchi et al., 1988). These effects on aldosterone synthesis are similar to that seen with ANF (Kudo and Baird, 1984). Therefore, it is clear that BNP antagonizes major components of the renin-angiotensin-aldosterone-system (RAAS).

BNP has also been shown to inhibit the production and secretion of another potent vasoconstrictor, ET-1, from endothelial cells (Emori et al., 1993) and cultured vascular smooth muscle cells (Hanehira et al., 1997) in a cGMP-dependent manner.

## **Natriuretic Peptides in Cardiovascular Disease and Heart**

### **Transplantation**

Under certain pathophysiological conditions affecting the cardiovascular system such as congestive heart failure (Burnett, Jr. et al., 1986), myocarditis (Takemura et al., 1995), and following heart transplantation (Masters et al., 1993; Atonu and Boateng, 1998; Masters et al., 1999), synthesis and release of both ANF and BNP is significantly augmented from both the atrial and ventricular cardiocytes.

In conditions of chronic volume overload-induced hypertrophy, BNP expression and release increases so dramatically that circulating levels have been shown in certain cases to exceed those of ANF (Mukoyama et al., 1991a). In fact, increased levels of ventricular production of the natriuretic peptides are hallmarks of cardiac hypertrophy and failure (Yokota et al., 1995). In response to either chronic pressure and volume overload *in vivo* (Izumo et al., 1988) or in cultured neonatal rat ventricular myocytes treated with hypertrophic agonists such as PE (Knowlton et al., 1991), ANG-II (Baker and Aceto, 1990; Sadoshima and Izumo, 1993) and ET-1 (Shubeita et al., 1990; Ito et al., 1991) the cardiac hypertrophy that develops is characterized by the re-expression of a cardiac fetal growth program. This fetal growth program consists of a number of phenotypic changes, including the activation of the IEGs and late activating genes such as the ANF and BNP genes (van Bilsen and Chien, 1993).

In human cardiac transplantation, ANF plasma levels remain elevated even though cardiac filling pressures, plasma renin activity (PRA) and plasma aldosterone levels begin to return towards physiological levels (Masters et al., 1993). In recent

studies plasma BNP levels remain highly upregulated despite replacement of the failing ventricle following transplantation (Masters et al., 1999). Unlike the cardiocyte hypertrophic process, the molecular basis for increased natriuretic peptide synthesis seen following cardiac allograft transplantation is unknown. Furthermore, in the same study by Masters et al. 1999, it was reported that BNP plasma levels, unlike ANF further increased prior to and peaked during significant rejection episodes as diagnosed by endomyocardial biopsy (Masters et al., 1999). Thus it appears that during human cardiac allograft rejection, the increase in natriuretic peptides is restricted to BNP only.

## Cytokines in Heart Transplantation

Orthotopic cardiac transplantation is presently the most effective and definitive treatment for end-stage cardiac failure in humans. The process of allograft rejection is controlled by a genetic system called the major histocompatibility complex (MHC) that encodes allelic forms of many immunogenic proteins. The rejection process begins with the presentation of these alloantigens associated with the MHC class II molecules on antigen presenting cells (APCs) present on the foreign organ (direct rejection pathway) or APCs present in the recipient's own lymphoid tissue (indirect rejection pathway). Allorecognition results from amino acid differences between donor and recipient MHC class II molecules that are recognized by the T-cell receptor (TCR) of resting recipient T helper lymphocytes ( $T_H$ -lymphocytes). The  $T_H$ -lymphocytes then initiate a cascade of events that result in cytokine production by these immune cells. Cytokines such as IL-2, IL-4 and IFN- $\gamma$  are involved in clonal expansion and activation of  $CD4^+$  ( $T_H$ ) and  $CD8^+$  (Natural Killer, NK) lymphocytes and B lymphocytes. IFN- $\gamma$  and TNF- $\alpha$  up-regulate expression of MHC I and II antigens and they also increase expression of adhesion molecules (Cunningham et al., 1994).

Numerous attempts by investigators have been made to determine if there exists a positive correlation between serum cytokine levels detected by ELISA, or cytokine mRNA by semi-quantitative RT-PCR and the level of rejection in the allograft. Currently, there is no consensus with respect to serum cytokine levels and the diagnosis of rejection (Grant et al., 1996). This may be because cytokines are local mediators and serum levels may not accurately reflect *in situ* levels at the graft. However, by RT-PCR, several observations indicate that there is an enhanced gene

expression of both pro-inflammatory cytokines IL-1 $\beta$ , IL-6, TNF- $\alpha$  and the immunoregulatory cytokines IL-2, IL-4 and IFN- $\gamma$  during the acute phase of cardiac allograft rejection (Duquesnoy and Demetris, 1995).

## AIMS OF THE STUDY

The aim of this study was to examine the molecular mechanisms and pathways that may be responsible for mediating the selective upregulation of BNP gene expression seen during acute cardiac transplant rejection in humans. To accomplish this, neonatal rat ventricular cardiocyte cell (NRVC) cultures were used to test the effect of individual pro-inflammatory cytokines (IL-1 $\beta$ , IL-2, IL-6, IFN- $\gamma$  and TNF- $\alpha$ ) on ANF and BNP gene expression and secretion. In order to circumvent the omission of individual cytokines, a mixed lymphocyte reaction (MLR) was generated and the conditioned medium from these allo-activated lymphocyte cultures was used to stimulate natriuretic peptide synthesis from NRVC cultures. Lastly, attempts were made to characterize important cardiocyte signaling pathways used by pro-inflammatory cytokines to selectively modulate ANF and BNP gene expression and secretion.

## MATERIALS AND METHODS

### *Neonatal rat ventricular cardiocyte cultures:*

Neonatal rat ventricular cardiocytes are easier to grow than their adult counterparts but also display the characteristic phenotype of limited proliferative capacity, preservation of receptor expression, natriuretic peptide production and secretion that develops during many pathophysiological conditions such as cardiac inflammation or hypertrophy (Gardner et al., 1988b; Argentin et al., 1994). The NRVC cultures used in this study are an established *in vitro* model system used by many different groups for examining cardiocyte gene expression (Knowlton et al., 1991; Shubeita et al., 1992b).

The experimental protocol was approved by the University of Ottawa animal care committee in accordance with the Canadian council on animal care guide to the care and use of experimental animals. The cardiocyte isolation is a modified procedure of Argentin *et al.* (Argentin et al., 1994). The apical 1/3 of the ventricles from 2 to 4 day old Sprague Dawley rats were isolated, washed in Joklik Modified MEM (Gibco), minced into 1mm or less fragments and subjected to four sequential 15 min digestions at 37°C in 0.1% collagenase (Worthington). Digestions were stopped by addition of FBS (Gibco) to a final concentration of 28.5%. The first digestion was discarded while the remaining digestions were pooled and filtered through a 100 µm filter. Filtrate was centrifuged at 200 x g for 5 min and the pellet resuspended in 10% FBS in DMEM (Gibco). Two 15 min pre-plating steps were used to eliminate fibroblast contamination. Unattached cardiocyte-enriched cells were counted and the

viability was assessed using trypan blue dye exclusion. The cells were then seeded onto Primaria<sup>®</sup> (Falcon) 6-well plates at a density of  $5 \times 10^4$  cells/cm<sup>2</sup> maintained in 10% FBS in DMEM at 37°C under 5% CO<sub>2</sub> and 95% humidified air for 16 h. The cells were washed twice in serum-free medium and replaced by serum-free DMEM/F12 basal medium supplemented with insulin-transferrin-selenium (5 µg/ml; 5 µg/ml; 5 ng/ml respectively) (Collaborative Research), and antibiotics (penicillin, 100 IU/ml and streptomycin, 100 µg/ml) (Sigma) for a 24 h equilibration period. This period was followed by the addition of numerous agonists and inhibitors.

***Treatment with cytokines, pro-hypertrophic agonists, MAP kinase and NF-κB inhibitors and cytokine receptor antagonists:***

Agonists including recombinant rat cytokines IL-1β, TNF-α, IL-6, IL-2, and IFN-γ (R&D Systems) at 0.5 to 100 ng/ml, ET-1 (Peninsula Laboratories) at 10 nM or vehicle (10 mM PBS with 0.1% BSA and H<sub>2</sub>O respectively) were added to the cultures for a period of 24 or 48 hours. In experiments using actinomycin D and cycloheximide, cultured cardiocytes were pre-incubated for 1 hour with vehicle (DMSO), actinomycin D (10 µg/ml) or cycloheximide (10 µg/ml) prior to the addition of IL-1β or TNF-α.

In experiments where MAP kinase inhibitors were used, SB203580 a specific p38 MAP kinase inhibitor at 1, 10 or 50 µM (Sigma), U0126 a specific MEK1/2 inhibitor at 10 µM (New England Biolabs) or vehicle (0.1% v/v DMSO) was added to the cardiocytes for 1 hour prior to cytokine stimulation.

NF- $\kappa$ B activity was suppressed with a cell-permeable inhibitory peptide, SN50 at 25  $\mu$ g/ml (Biomol). SN50, and an inactive mutant control peptide, SN50M was used as a control and both these peptides were added 1 hour prior to cytokine stimulation.

Functional inhibition of pro-inflammatory cytokine IL-1 $\beta$  and TNF- $\alpha$  signaling in SF-MLR-CM was accomplished by pre-incubating NRVCs with an IL-1 receptor antagonist (IL-1RA) at 1  $\mu$ g/ml or a combination of soluble TNF receptor I/II antagonists (sTNF-RI/II) (both from R&D systems) at 1  $\mu$ g/ml for 1 hour prior to the addition of SF-MLR-CM. The concentration of IL-1RA used in this study was based on reports published by other investigators which have demonstrated that concentrations of IL-1RA of 500 ng/ml successfully inhibited all rat IL-1 $\beta$ -induced iNOS activity but at the same time, did not affect isoproterenol-stimulated cardiocyte contractile function (Ungureanu-Longrois et al., 1995). The concentration of sTNF-RI/RII used was based on the ED<sub>50</sub> which is 1-3  $\mu$ g/ml to inhibit cytotoxicity of the mouse L-929 cell line caused by 100 pg/ml of TNF- $\alpha$  according to data provided by the manufacturer.

***Mixed lymphocyte reaction:***

The rat lymphocyte isolation and MLR procedures were essentially those of Murase *et al.* (Murase et al., 1993) using the serum-free medium described by Gersten and Cohn (Gersten and Cohn, 1985). Two inbred rat strains, Brown Norway (#BN/CRLBR) and Lewis rats (#Lew/CRLBR) (Charles River, Quebec) were used. Under sterile conditions, cervical lymph nodes were isolated and placed in ice cold wash medium; RPMI-1640 (Gibco) supplemented with 100  $\mu$ M non-essential amino

acids (Gibco), 100  $\mu$ M sodium pyruvate (Gibco), 1 mM L-glutamine (Gibco),  $5.5 \times 10^{-2}$  M 2-mercaptoethanol (Sigma), 100 IU/ml penicillin, and 100  $\mu$ g/ml streptomycin (Sigma). Lymph nodes were ground up by a #60 mesh sieve cup held over a 100-mm petri dish to dislodge the lymphocytes. The resultant suspension was centrifuged at 150 x g for 10 minutes. The supernatant was decanted slowly in a continuous motion and the pellet was resuspended by trituration with a disposable transfer pipette and washed again with wash medium followed by centrifugation. The pellet was resuspended in 10 ml of serum-free lymphocyte medium (SF-LM: Iscoves medium (Gibco); supplemented with essential amino acids,  $5 \times 10^{-5}$  M 2-mercaptoethanol, 10  $\mu$ g/ml bovine insulin, 10  $\mu$ g/ml human transferrin, 2 mM L-glutamine and 50  $\mu$ g/ml gentamicin). Cell suspension aliquots were diluted 1:1 with 0.25% eosin stain and counted using a haemocytometer. Lymphocytes are nucleated and only a few erythrocytes are present using this methodology. Viability using eosin dye was estimated to be around 90%. The Lewis rats were arbitrarily assigned to be the responder cells and were diluted to  $10 \times 10^6$  cells/ml in SF-LM. Whereas, the Brown Norway rats were assigned as the stimulator cells and were plated at  $2.5 \times 10^6$  cells/ml in SF-LM. Stimulators were  $\gamma$ -irradiated with 2000 rads from a x-ray source to inhibit DNA synthesis in the stimulators. The ratio of responders to stimulator was 4:1. Cells were transferred to a T-75 flask and incubated for 5 days at 37<sup>0</sup>C in 5% CO<sub>2</sub> and 95% humidified air. Supernatant from a 5 day MLR was collected by decanting to remove cells, followed by filtration through a 0.22  $\mu$ m filter to remove additional debris. For the analysis of [<sup>3</sup>H]-thymidine incorporation: 100  $\mu$ l of stimulators ( $1 \times 10^5$ ) and 100  $\mu$ l of responders ( $2 \times 10^5$ ) were plated into a 96-well round bottom microplate and incubated for 4 days after which cells were pulse-labeled with 1  $\mu$ Ci

[<sup>3</sup>H]-thymidine. After an overnight incubation cells were harvested to determine [<sup>3</sup>H]-thymidine uptake.

Freshly isolated cardiocytes pre-equilibrated in serum-free cardiocyte medium for 24 hours were treated with serum-free mixed lymphocyte reaction conditioned medium (SF-MLR-CM) in 0, 10, 20, 50, and 100% proportions for 48 hours. The experiments were compared against serum-free lymphocyte medium (SF-LM), along with non-activated responder and stimulator conditioned medium controls. Natriuretic peptides released into the medium were measured using the RIA.

***Bacterial transformation and plasmid preparation:***

The -2.2 kbp rat BNP promoter coupled to a luciferase reporter gene of the pXP2 vector (Nordeen, 1988; Grepin et al., 1994) was a kind gift from Dr. Mona Nemer (Institut de Recherches Cliniques de Montréal). To clone the BNP promoter-luciferase construct, 10 pg of cDNA was used to transform INVαF' competent cells with a One Shot™ kit as per manufacturer's instructions (Invitrogen).

The isolation and purification of cDNA was accomplished with the EndoFree™ Plasmid Maxi kit (QIAGEN®). The identity of the clone was confirmed by restriction digestion with *BglII/SmaI* and partial nucleotide sequencing with the BigDye™ Terminator kit (ABI-Prism 310, Perkin-Elmer Sciences).

***Transfection of rat -2.2 kbp BNP promoter:***

In a microcentrifuge tube, 1.0 µg of -2.2-kbp BNP promoter plasmid or pGL3 luciferase SV40 promoter internal control plasmid (Promega) along with 0.5 µg of

pSV- $\beta$ -galactosidase expression plasmid (Promega) was mixed with 4.5  $\mu$ l Fugene 6<sup>®</sup> (Roche) transfection reagent in 100  $\mu$ l serum-free DMEM/F12 medium and incubated for 45 min at room temperature. The transfection complex was added dropwise to the 1 day old neonatal rat cardiocyte cultures plated at a density of  $1 \times 10^5$  cells/cm<sup>2</sup> on 6- or 24-well Primaria<sup>®</sup> culture dishes with 10% FBS in DMEM and incubated for 6-hours at 37<sup>0</sup>C in 5% CO<sub>2</sub> and 95% humidified air. The Fugene 6<sup>®</sup> transfection complex transfected approximately 5% of total cells as determined by  $\beta$ -galactosidase histochemical staining (See below). After the transfection, the medium was then changed to serum-free DMEM/F12 for a 24-hour equilibration period followed by stimulation with IL-1 $\beta$  or TNF- $\alpha$ . For detection of luciferase and  $\beta$ -galactosidase activities, cardiocytes were lysed with 150  $\mu$ l of lysis buffer (100 mM potassium phosphate (pH 7.8), 0.2 % triton X-100 and 0.5 mM DTT). Extracts were then assayed according to the manufacturer's instructions using the dual-light<sup>®</sup> system for chemiluminescent reporter gene assay system (Applied Biosystems) and the signal was detected using a POLARstar Galaxy<sup>®</sup> microplate reader (BMG labtechnologies). Luciferase activity was divided by  $\beta$ -galactosidase ( $\beta$ -gal) activity per well to normalize for transfection efficiency.

***$\beta$ -Galactosidase histochemical staining:***

To monitor  $\beta$ -galactosidase activity in cardiocytes transfected with a pSV- $\beta$ -galactosidase expression plasmid, 2-day old NRVCs plated onto 35 mm 6-well multiplates were washed with PBS at room temperature and then fixed with 0.2% glutaraldehyde in PBS (Sigma) for five minutes at room temperature. The fixative was aspirated and the cells were washed twice with 2 ml PBS. Cells were then stained for 4 hours at 37<sup>0</sup>C in a humidified incubator with staining solution (20 mg/ml

X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactoside) (Sigma) in N, N-dimethylformamide (Sigma); 5 mM potassium ferricyanide; 5 mM potassium ferrocyanide; 2 mM MgCl<sub>2</sub> in PBS). Wells were aspirated and stained cardiocytes were viewed under brightfield microscopy with a Leitz Labovert<sup>®</sup> inverted microscope and photographed with a Leitz vario Orthomat<sup>®</sup> 2 camera coupled to a Leitz WILD MPS46/52 photosystem.

***Radioimmunoassay:***

The RIAs were performed using the double-antibody method as described by Sarda et al. (Sarda et al., 1989). ANF and BNP standard curves were generated using rat ANF<sub>99-126</sub> or BNP<sub>64-95</sub> peptides with concentrations ranging from 31.25, 62.50, 125.0, 250.0, 500.0 and 1000.0 pg/ml in RIA buffer (0.1 M sodium phosphate; 0.05 M NaCl; 0.01% sodium azide; 0.1% Triton X-100; 0.1% BSA (heat treated)). 100 µl of ANF/BNP standards or samples was mixed with 100 µl of ANF or BNP antiserum at a dilution of 1:100,000 and incubated in the dark at 4°C for 4 or 24 hours respectively. Following the incubation, 100 µl of iodinated ANF<sub>99-126</sub> or BNP<sub>64-95</sub> (10,000 counts/minute) was added to the standards or samples and incubated in the dark at 4°C for 24 hours. Following this incubation, goat anti-rabbit gamma globulin (Advance Chemtech) and normal rabbit serum (100 µl) was added to the tubes and incubated at room temperature for 2-hours. 1.5 ml polyethylene glycol (6.25%) was added and the tubes were centrifuged at 2000xg. The supernatant was discarded and the pellets were counted using a γ-counter (1272 CliniGamma, LKB Wallac).

### ***RNA extraction and Northern blot:***

Natriuretic peptide gene expression was determined using Northern blot analysis (Yokota et al., 1995). Total RNA was isolated with TRIzol<sup>®</sup> (Gibco BRL) reagent, a mono-phasic solution of phenol and guanidine isothiocyanate by a modified method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Briefly, cardiocyte cultures grown on 35 mm 6-well plates were lysed with 1 ml of TRIzol<sup>®</sup> and the cell lysates were kept at -80°C. Phase separation was accomplished with 0.2 ml of chloroform and the aqueous phase was then mixed with 1 ml of isopropyl alcohol. The RNA was then washed with 2 cycles 75% ethanol. The final pellet was resuspended in sterile diethylpyrocarbonate (DEPC)-treated water and the RNA concentration was measured by spectrophotometry at 260 and 280 nm. A 5 µg RNA sample was denatured in 50% formamide, 7% formaldehyde, 1X MOPs (10X MOPS buffer: 0.2 M 3-[N-Morpholino]propanesulfonic acid; 100 mM Na acetate pH 7.0; 20 mM ethylenediaminetetraacetic acid (EDTA)) and 0.1% bromophenol blue. The samples were then electrophoresed in a denaturing 1.5% agarose-0.22 M formaldehyde gel. The RNA was transferred to a nylon membrane (Hybond-N<sup>+</sup>, Amersham Biosciences) by capillary action overnight using 10X standard saline citrate (20X SSC; 3 M NaCl, 0.3 M Na citrate, pH 7.0). The RNA was crosslinked to the membrane by UV irradiation and the membrane was pre-hybridized with ULTRAhyb<sup>™</sup> hybridization buffer at 68°C for 30 minutes. Membranes were hybridized for 17-20 hours at 42°C with a radiolabeled 900 bp EcoRI/HindIII fragment of full-length rat ANF cDNA (Flynn et al., 1985) or 595 bp Sall fragment of rat BNP cDNA (Bruneau and de Bold, 1994) along with a 2 kb BamHI/BglIII fragment of the mouse phosphoglycerate kinase (PGK) (Mori et al., 1986) cDNA that served as an internal control. The cDNA probes were labeled with [ $\alpha$ -<sup>32</sup>P]dCTP in a

random-priming oligodeoxyribonucleotide reaction using Ready-To-Go™ DNA labeling beads (Amersham Biosciences). Unincorporated nucleotides were removed with ProbeQuant™ G-50 Micro Column (Amersham Biosciences) prior to hybridization. Following hybridization, the blots were washed twice for 10 minutes in 2X SSC, 0.1% SDS at 42°C followed by another two rounds of washing for 20 minutes with 0.1X SSC, 0.1% SDS at 55°C. Membranes were exposed to an intensifying phosphor screen (Kodak) which was subsequently scanned with the Personal Molecular Imager™- FX system (Bio Rad) and analyzed with Quantity One™ software (Bio Rad).

***Western blot and kinase activity assays:***

Cardiocytes were lysed 15 minutes following cytokine treatment using cell lysis buffer (20 mM Tris (pH 7.5); 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 2.5 mM sodium pyrophosphate; 1 mM  $\beta$ -glycerophosphate; 1 mM  $\text{Na}_3\text{VO}_4$ ; 1  $\mu\text{g}/\text{ml}$  leupeptin; 1 mM PMSF) at 4°C and centrifuged at 12,000 rpm for 10 minutes as per manufacturer's instructions (New England Biolabs). An additional immunoprecipitation step was required for the *in vitro* kinase assay. 200  $\mu\text{g}$  of total protein was mixed with 20  $\mu\text{l}$  of immobilized phospho-specific p38 MAP kinase (Thr180/Tyr182) (New England Biolabs) monoclonal antibody and incubated overnight at 4°C as per manufacturer's instructions (New England Biolabs). After a series of washes in lysis buffer, the kinase reaction was initiated by the addition of 200  $\mu\text{M}$  ATP and 2  $\mu\text{g}$  of ATF-2 fusion protein and incubated at 30°C for 30 minutes. The reactions were terminated by the addition of 3X SDS sample buffer.

25 µg of protein (Quantified with Bio Rad detergent compatible protein assay Kit, Bio Rad) along with biotinylated protein markers were loaded on a 10% SDS-polyacrylamide gel and run in electrode buffer (25 mM Tris, 192 mM glycine pH 8.3, 1% SDS). Proteins were transferred to a PVDF membrane at 100 V for 1 hour in transfer buffer (25 mM Tris, 192 mM glycine pH 8.3) followed by blocking (Tris-buffered saline/TBS, 5% w/v non fat milk, 0.1% tween-20) for 1 hour at room temperature. Blots were incubated overnight at 4°C with phosphorylation specific p38 MAP kinase (Thr180/Tyr182) polyclonal antibodies (New England Biolabs) or in the case of the *in vitro* kinase assay, phosphorylation specific ATF-2 (Thr71) polyclonal antibodies (New England Biolabs) at 1:1000 in primary antibody dilution buffer (TBS, 5%BSA, 0.1% tween-20). After washing with TBS/T, blots were incubated in blocking buffer with HRP-conjugated secondary antibody (1:2000) and HRP-conjugated anti-biotin antibody (1:1000) for 1 hour at room temperature. Development of the blots was accomplished using enhanced ECL (New England Biolabs) and autoradiography. Protein densities were quantified with Quantity One™ software (Bio Rad). To determine total unphosphorylated MAP kinase protein PVDF membranes were incubated in stripping solution (62.5mM Tris-HCl, 2% SDS, 100mM 2-mercaptoethanol) for 30 minutes at 50°C, rinsed in TBS/T and reprobbed with polyclonal p38 MAP kinase antibodies (New England Biolabs). As a control for total ATF protein, cell extracts were obtained as outlined, but without the immunoprecipitation step and a non-phosphorylation specific ATF-2 polyclonal antibody at 1:1000 dilution was used as a control (New England Biolabs).

***Enzyme-linked immunosorbent assay (ELISA):***

IL-1 $\beta$  and TNF- $\alpha$  levels in SF-MLR-CM obtained from 5 day MLR cultures were examined using the Quantikine<sup>®</sup> (R & D Systems). The system is a sandwich ELISA technique where a microplate is pre-coated with an immobilized (rat IL-1 $\beta$  or TNF- $\alpha$ ) antibody that binds any applied samples, standards or controls. A horseradish peroxidase-linked polyclonal (either rat IL-1 $\beta$  or rat TNF- $\alpha$ ) antibody was applied to the wells and a series of washes were performed. A hydrogen peroxide substrate with a chromogen (3,3',5,5'-tetramethylbenzidine) was then added where the colour produced is proportional to the amount of cytokine present. In brief, supernatants from the 5 day MLR cultures were collected, centrifuged (1000xg for 15 minutes) and immediately frozen in individual sample sized aliquots at -20°C prior to the beginning of the assays. A standard curve was generated using rat IL-1 $\beta$  or TNF- $\alpha$  calibration standards. A 50  $\mu$ l sample, standard or control (IL-1 $\beta$  or TNF- $\alpha$  buffered protein) was mixed with 50  $\mu$ l of assay diluent in a 96 well multi-plate and incubated for 2 hours at room temperature. Each well was washed five times with a Wash buffer followed by the addition of anti-rat IL-1 $\beta$  or anti-TNF- $\alpha$  conjugate and the microplates were incubated for another 2 hours at room temperature. Five washing steps were followed by the addition of the substrate and an incubation of 30 minutes in the dark at room temperature. A Stop solution was added to terminate the reaction and the optical density was determined by a spectrophotometer (Bio-Rad model 3550-UV) at 450 nm with a wavelength correction set at 595 nm.

***Statistics:***

One way ANOVA followed by Bonferroni post-test using Graphpad Prism 3.0<sup>®</sup> software was used for statistical analysis. A value of  $p \leq 0.05$  was considered significant. Values are expressed as mean  $\pm$  SEM.

## RESULTS

### **IL-1 $\beta$ and TNF- $\alpha$ Selectively Increase BNP Secretion**

Figures 4A and 4B show BNP and ANF secretion from neonatal rat ventricular cardiocytes (NRVCs) cultured in serum-free medium after exposure to IL-1 $\beta$  or TNF- $\alpha$  for 24 hours. BNP secretion was significantly increased with respect to vehicle using 0.5 ng/ml of IL-1 $\beta$  for 24 hours (141.9 $\pm$ 9.3 pg/ml versus 79.9 $\pm$ 10.0 pg/ml). Additional increases of IL-1 $\beta$  to 1 ng/ml and 10 ng/ml further increased BNP levels (205.4 $\pm$ 11.5 pg/ml and 193.3 $\pm$ 21.6 pg/ml, respectively). Similarly, treatment with TNF- $\alpha$  at concentrations of 1 ng/ml and 20 ng/ml also increased BNP secretion in the medium (208.9 $\pm$ 13.3 pg/ml and 167.3 $\pm$ 19.9 pg/ml). Neither IL-1 $\beta$  nor TNF- $\alpha$  caused a change in ANF secretion at 24 hours with respect to vehicle (529.2 $\pm$ 30.0 pg/ml). An additional experiment was performed with endothelin-1 (ET-1), an established stimulator of ANF and BNP secretion to determine if our NRVCs were capable of secreting ANF. Unlike the pro-inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$ , stimulation with ET-1 (10 nM) increased the secretion of ANF (2523.2 $\pm$ 56.1 pg/ml) (Figure 4B) as well as BNP (772.5 $\pm$ 38.0 pg/ml) (Figure 4A).

Figures 4C and 4D show BNP and ANF secretion into the medium after exposure to IL-1 $\beta$  or TNF- $\alpha$  for 48 hours. BNP secretion was significantly increased by IL-1 $\beta$  at 0.5, 1 and 10 ng/ml (273.7 $\pm$ 17.8, 324.6 $\pm$ 25.3 and 386.8 $\pm$ 20.9 pg/ml) as compared to the vehicle control (167.2 $\pm$ 10.3 pg/ml). TNF- $\alpha$  at 1 and 20 ng/ml also increased BNP levels (378.8 $\pm$ 44.2 and 482.0 $\pm$ 37.2 pg/ml). However, even after 48

hours of stimulation, ANF levels were not significantly different with respect to vehicle with any dose of either IL-1 $\beta$  (0.5, 1 and 10 ng/ml) or TNF- $\alpha$  (0.5, 1 and 20 ng/ml).

### **IL-1 $\beta$ and TNF- $\alpha$ Specifically Upregulate BNP mRNA**

Figure 5 shows BNP and ANF steady state mRNA levels in cardiocytes after 48 hours of treatment with IL-1 $\beta$  or TNF- $\alpha$ . BNP mRNA levels were significantly increased by both IL-1 $\beta$  (10 ng/ml) and TNF- $\alpha$  (20 ng/ml) after 48 hours (Figure 5A). The increase in BNP mRNA is due to an increase in transcription as pre-treatment with actinomycin D (a transcriptional inhibitor) at 10  $\mu$ g/ml completely suppressed the IL-1 $\beta$  and TNF- $\alpha$ -mediated increase in BNP mRNA (Figure 5A). By contrast, ANF mRNA is not altered after exposure for 48 hours of treatment with IL-1 $\beta$  (10 ng/ml) or TNF- $\alpha$  (20 ng/ml) (Figure 5B). Treatment of the cardiocytes with cycloheximide (protein synthesis inhibitor) at 10  $\mu$ g/ml failed to alter cytokine-stimulated increases in BNP mRNA (Figure 5C) and basal ANF mRNA (Figure 5D).

### **IFN- $\gamma$ Increases IL-1 $\beta$ Induced Stimulation of BNP Secretion**

In addition to IL-1 $\beta$  and TNF- $\alpha$ , many other cytokines are believed to be present and important in mediating the cardiac allograft rejection process. The most likely candidates are IL-6, IL-2 and IFN- $\gamma$ . As shown in Figure 6, treatment of cardiocytes with IL-6 (5, 25 and 100 ng/ml), IL-2 or IFN- $\gamma$  (500 pg/ml, 5, 25, 100 ng/ml) individually for 48 hours did not significantly increase either BNP (Figure 6A) or ANF (Figure 6B) secretion with respect to vehicle controls. However, the amount of BNP released by stimulation by both IFN- $\gamma$  (0.5 ng/ml) and IL-1 $\beta$  (5 ng/ml) in

combination was significantly greater than that of IL-1 $\beta$  alone (Figure 7A). This effect was specific for BNP, as ANF secretion was not affected by the addition of this combination of cytokines (Figure 7B).

### **Allo-Activated serum-free MLR Conditioned Medium Specifically increases BNP Secretion**

Systemic effects of immune activation are often a result of sequential production and concerted action of a plethora of pro-inflammatory cytokines on target tissue or cells. In order to circumvent the possible omission of specific cytokines a novel methodological application of a recognized *in-vitro* model of transplantation immunity was utilized. In the mixed lymphocyte reaction (MLR), lymphocytes from two different rat strains are combined, producing an allogenic reaction resulting in cytokine production and cell proliferation. Cell proliferation can be measured by <sup>3</sup>[H]-thymidine incorporation and the results are shown in figure 8. Only the combination of responder and stimulator lymphocytes in the MLR results in cell proliferation.

To examine the effects of the serum-free MLR conditioned medium (SF-MLR-CM) harvested from 5-day old MLR cultures on natriuretic peptide secretion, conditioned medium in 10, 20, 50 and 100% proportions was added to the cardiocyte cultures and the effects were measured 48 hours later. As shown in figure 9A, BNP release was markedly and dose dependently increased by the SF-MLR-CM at 20, 50 and 100% proportions as compared to SF-LM conditioned medium at their respective proportions ( $p < 0.001$ ) or the responder and stimulator conditioned medium controls ( $P < 0.001$ ). However, ANF concentration in the medium after SF-MLR-CM treatment

at 48 hours was not significantly different from the serum-free lymphocyte medium (SF-LM), or from responder and stimulator conditioned medium controls (Figure 9B). Furthermore, as shown in figure 9A, pre-treatment with SB203580 (1  $\mu$ M) for 1 hour completely abolished the 100% SF-MLR-CM induced increase in BNP secretion.

### **Inhibitors to IL-1 $\beta$ and TNF- $\alpha$ receptors do not decrease serum-free MLR conditioned medium mediated increase in BNP secretion**

To determine if functional inhibition of pro-inflammatory cytokine IL-1 $\beta$  and TNF- $\alpha$  signaling would alter SF-MLR-CM-mediated increases in BNP secretion cultured cardiocytes were pre-incubated with an IL-1 receptor antagonist (IL-1RA) or a combination of soluble TNF receptor I/II antagonists (sTNF-RI/II). As shown in figure 10A, neither IL-1RA (1  $\mu$ g/ml) nor sTNF-RI/II (1  $\mu$ g/ml) suppressed SF-MLR-CM induced increases in BNP secretion. These receptor antagonists also did not alter basal ANF secretion (Figure 10B).

### **IL-1 $\beta$ and TNF- $\alpha$ are not present in serum-free MLR conditioned medium by ELISA**

To determine if detectable concentrations of IL-1 $\beta$  and TNF- $\alpha$  were present in the serum-free MLR conditioned medium, supernatant from a 5-day MLR was collected and examined by ELISA (Quantikine<sup>®</sup> M, R&D Systems). The minimum detectable amounts of rat IL-1 $\beta$  and TNF- $\alpha$  by this kit according to the manufacturer is 5 pg/ml. Our results show that no detectable amounts of IL-1 $\beta$  or TNF- $\alpha$  were present in the SF-MLR-CM.

### **IL-1 $\beta$ and TNF- $\alpha$ Rapidly Activate p38 MAP Kinase**

Treatment with either IL-1 $\beta$  or TNF- $\alpha$  for 15 minutes significantly increased phosphorylated p38 MAP kinase abundance versus the vehicle control as determined by western blot (Figure 11A). To examine if there was an increase in p38 activity, IL-1 $\beta$  or TNF- $\alpha$  stimulated cardiocyte extracts were subjected to immunoprecipitation by an immobilized p38 monoclonal antibody directed against phosphorylated p38 (Thr180/Tyr182). Immunoprecipitated extracts were then subjected to an *in vitro* kinase assay with ATF-2 fusion protein as the p38 substrate. As shown in figure 11B, p38 MAP kinase activity is also significantly upregulated in response to 15 minutes of treatment with IL-1 $\beta$  or TNF- $\alpha$ .

### **p38 MAP Kinase Inhibition Significantly Decreases IL-1 $\beta$ and TNF- $\alpha$**

#### **Mediated BNP Gene Expression and Secretion from Neonatal Rat Ventricular Cardiocytes**

Pretreatment of cardiocytes with 10  $\mu$ M or 50  $\mu$ M SB203580 reduced IL-1 $\beta$ - and TNF- $\alpha$ -stimulated BNP secretion to basal levels (Figure 12A). However, this treatment did not alter basal ANF levels (Figure 12B). The reduction in IL-1 $\beta$ - and TNF- $\alpha$ -stimulated BNP release caused by SB203580 was accompanied by a decrease in BNP gene expression. As shown in Figure 13, the cytokine-mediated increase in BNP mRNA was significantly attenuated by pretreatment with SB203580 (Figure 13A). SB203580 had no effect on basal ANF mRNA levels (Figure 13B).

## **Inhibition of NF- $\kappa$ B does not decrease IL-1 $\beta$ - or TNF- $\alpha$ -mediated increase in BNP secretion**

To investigate the role of the NF- $\kappa$ B transcription factor in IL-1 $\beta$ - or TNF- $\alpha$ -mediated increase in BNP gene expression, a cell-permeable NF- $\kappa$ B inhibitory peptide, SN50 was used to inhibit NF- $\kappa$ B translocation and activation. SN50 (25  $\mu$ g/ml) at a concentration known to significantly inhibit NF- $\kappa$ B activity did not suppress IL-1 $\beta$  or TNF- $\alpha$ -induced increases in BNP secretion (Figure 14A). As a control, a mutant NF- $\kappa$ B inhibitory peptide, SN50M (25  $\mu$ g/ml) did not have an effect on BNP or ANF secretion (Figures 14A and 14B).

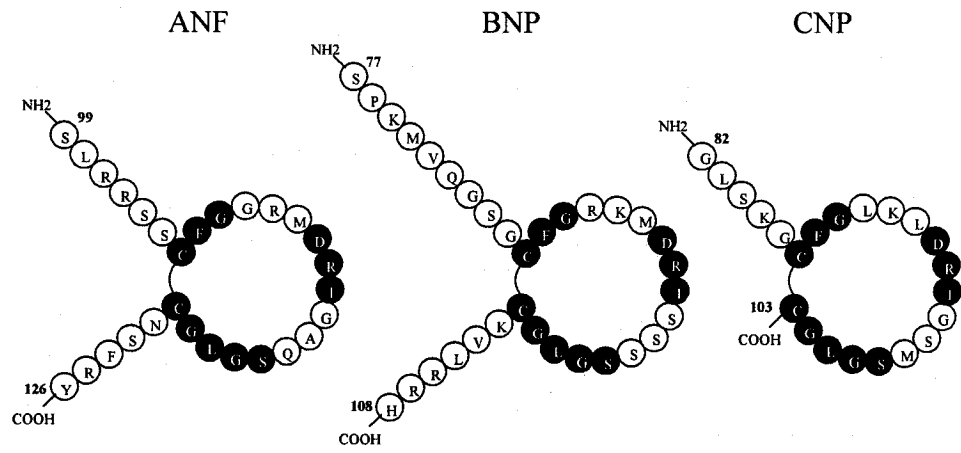
## **IL-1 $\beta$ and TNF- $\alpha$ can transactivate a -2.2-kbp rat BNP promoter**

To examine the ability of IL-1 $\beta$  or TNF- $\alpha$  to activate the BNP promoter, cultured cardiocytes were transfected with both a -2.2-kbp rat BNP promoter coupled to a luciferase reporter gene and a pSv- $\beta$ -galactosidase expression plasmid to normalize for transfection efficiency. An estimated 5% of total cardiocytes were transfected using the Fugene 6<sup>®</sup> method (Figure 15). Incubation with IL-1 $\beta$  (10 ng/ml) and TNF- $\alpha$  (20 ng/ml) significantly activated this promoter with respect to non-stimulated controls (Figure 16). Inhibition of p38 MAP kinase with SB203580 (10  $\mu$ M) significantly decreased IL-1 $\beta$ -mediated and reduced TNF- $\alpha$ -mediated activation of the BNP promoter. ET-1 (10 nM) was used as a positive control for transactivation of the BNP promoter, and complete inhibition of this activation was accomplished by pre-incubation with 10  $\mu$ M of the MEK 1/2 inhibitor, U0126, to inhibit the p44/p42 ERK MAP kinase pathway.

**Figure 1:**

The biologically active forms of the natriuretic peptides. Amino acids that are shared by all members of the natriuretic peptide family are identified by filled circles. A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine

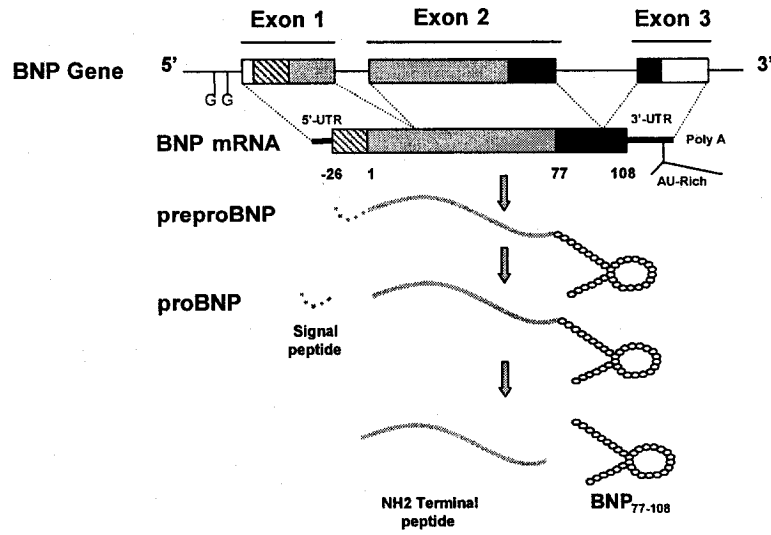
Figure 1



**Figure 2:**

Structure of the gene and biosynthetic pathway of human BNP. Solid black section are those that code for the mature BNP<sub>77-108</sub>. Grey section codes for NH<sub>2</sub>-terminal fragment and the stripped section codes for the signal peptide. Numbers describe the amino acid position relative to the sequence encoded in proBNP. G's, are the location of the GATAAA sequences. UTR, untranslated region.

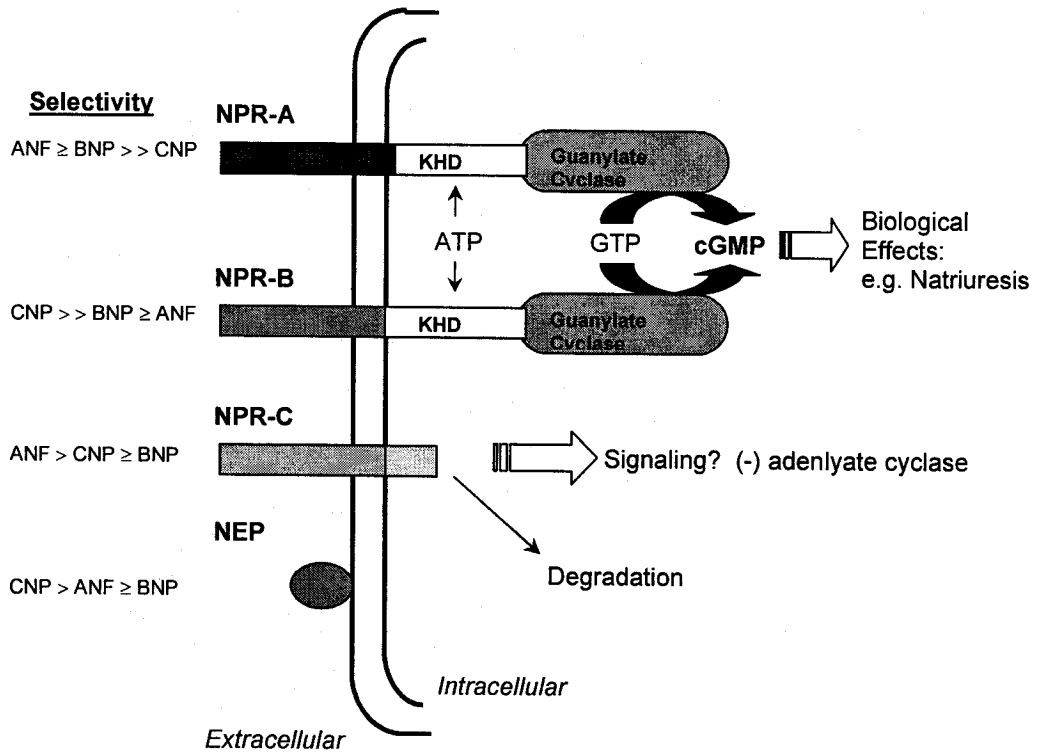
Figure 2



**Figure 3:**

The natriuretic peptide receptors. Binding of ANF, BNP to NPR-A stimulates intrinsic guanylyl cyclase activity. The process of ligand-binding to the generation of cGMP is ATP dependent and requires the kinase homology domain (KHD) portion of the receptor. NPR-A, natriuretic peptide receptor-A; NPR-B, natriuretic peptide receptor-B; NPR-C, natriuretic peptide receptor-C; NEP, neutral endopeptidase 24.11

Figure 3

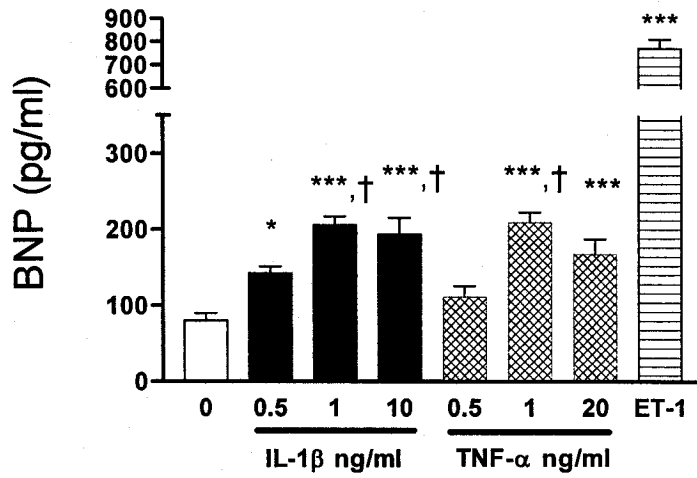


**Figures 4A, 4B, 4C and 4D:**

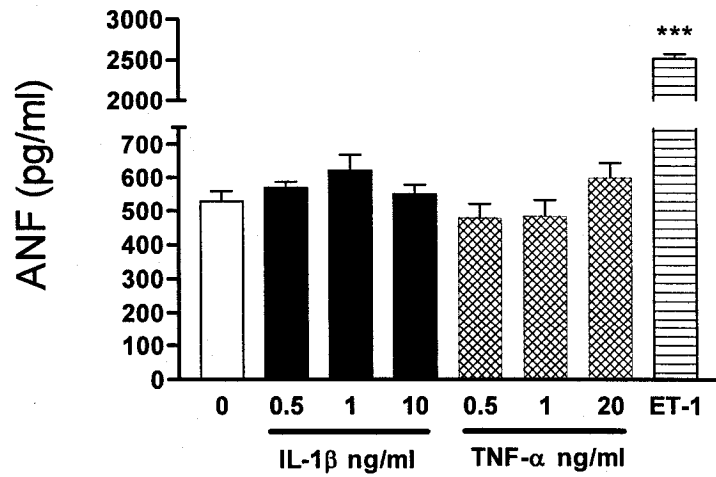
Natriuretic peptide secretion into medium by primary neonatal ventricular cardiocyte cultures stimulated by IL- $\beta$ , TNF- $\alpha$  or ET-1 for 24 hours (A and B) or 48 hours (C and D) as measured by radioimmunoassay. (A) BNP secretion stimulated by IL-1 $\beta$  (0.5, 1 and 10 ng/ml) TNF- $\alpha$  (0.5, 1 and 20 ng/ml) or ET-1 (10 nM) for 24 hours. (B) ANF secretion stimulated by IL-1 $\beta$ , TNF- $\alpha$  or ET-1 for 24 hours. (C) BNP secretion stimulated by IL-1 $\beta$  (0.5, 1 and 10 ng/ml) or TNF- $\alpha$  (0.5, 1 and 20 ng/ml) for 48 hours. (D) ANF secretion stimulated by IL-1 $\beta$  or TNF- $\alpha$  for 48 hours. Data are presented as means  $\pm$  SEM. \*, p<0.05; \*\*, p<0.01; \*\*\*, p<0.001 vs. control; †, p<0.05 vs 0.5 ng/ml concentration group; §, p<0.001 vs. 0.5 ng/ml concentration group (Statistical analysis by ANOVA). n=7 for all groups in triplicate.

Figures 4A and 4B

A

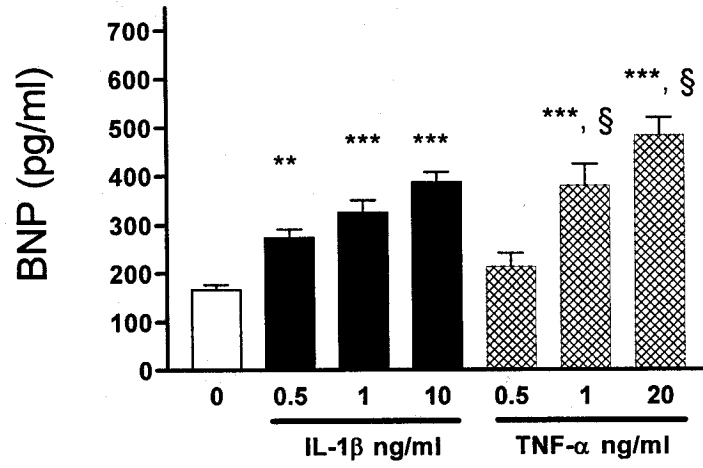


B

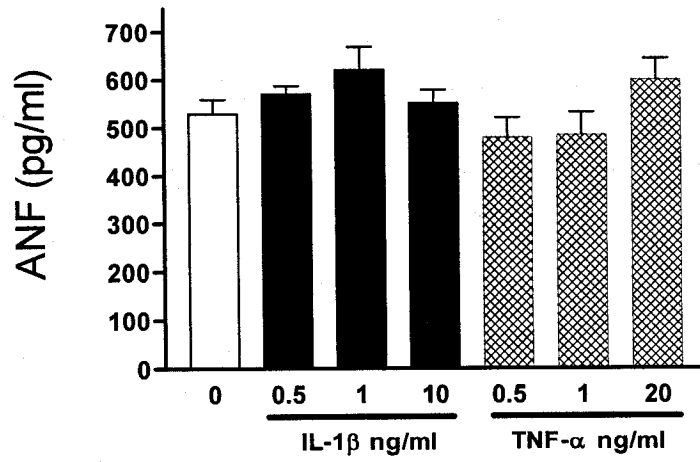


Figures 4C and 4D

C



D

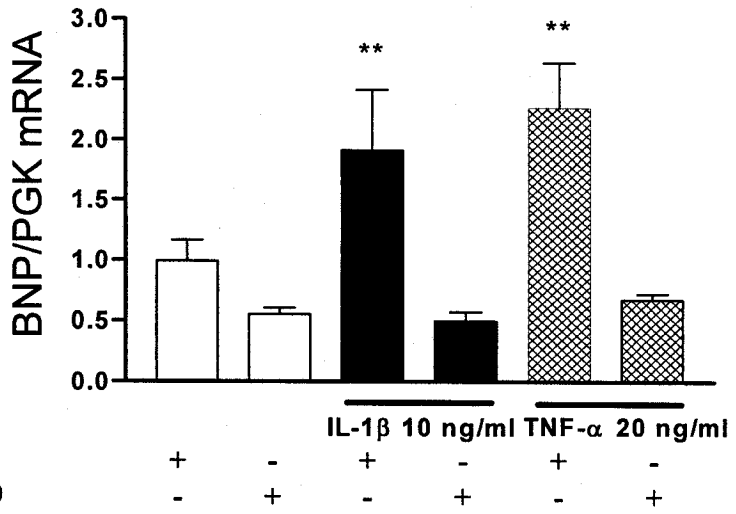
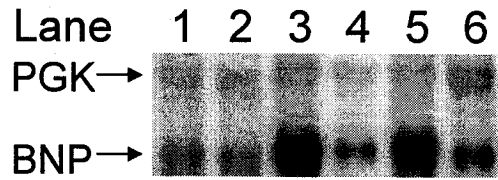


**Figures 5A, 5B, 5C and 5D:**

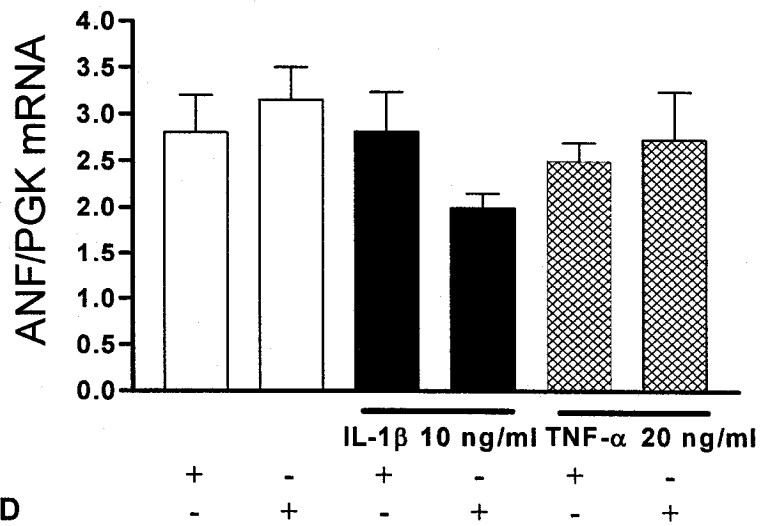
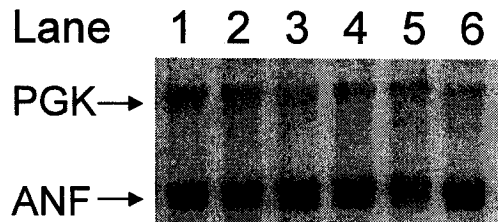
Representative Northern blot and densitometric analysis of ANF and BNP mRNA levels in primary neonatal ventricular cardiocyte cultures stimulated by IL-1 $\beta$  or TNF- $\alpha$  pre-incubated with actinomycin D (A and B) or cycloheximide (C and D) for 48 hours. Signals were normalized to phosphoglycerate kinase (PGK) mRNA. Cultured cardiocytes were pre-incubated for 1 hour with vehicle (DMSO) or actinomycin D (10  $\mu$ g/ml) as indicated, followed by stimulation with cytokines for 48 hours (A and B). (A) BNP/PGK mRNA in response to IL-1 $\beta$  (10 ng/ml) or TNF- $\alpha$  (20 ng/ml). (B) ANF/PGK mRNA in response to IL-1 $\beta$  (10 ng/ml) or TNF- $\alpha$  (20 ng/ml). Cultured cardiocytes were pre-incubated for 1 hour with vehicle (DMSO) or cycloheximide (10  $\mu$ g/ml) as indicated, followed by stimulation with cytokines for 48 hours (C and D). (C) BNP/PGK mRNA in response to IL-1 $\beta$  (10 ng/ml) or TNF- $\alpha$  (20 ng/ml). (D) ANF/PGK mRNA in response to IL-1 $\beta$  (10 ng/ml) or TNF- $\alpha$  (20 ng/ml). Data are presented as means  $\pm$  SEM. \*\*,  $p < 0.01$  vs. control. (Statistical analysis by ANOVA). Figure 5A and 5B,  $n = 5$  in triplicate. Figure 5C and 5D,  $n = 3$  in triplicate.

Figures 5A and 5B

A

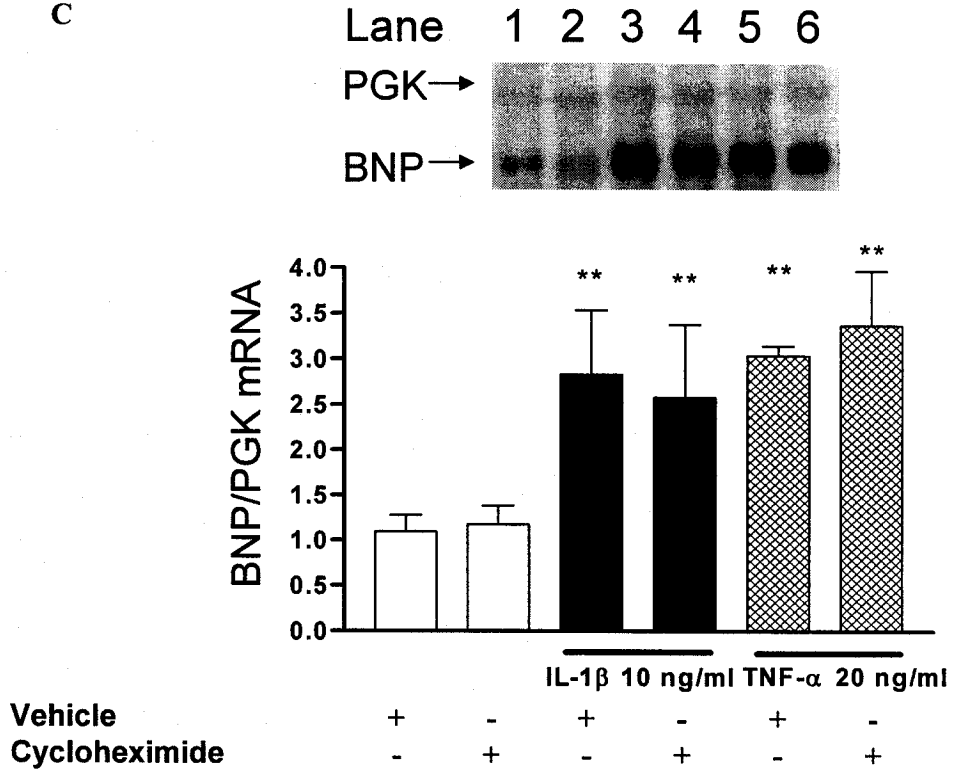


B

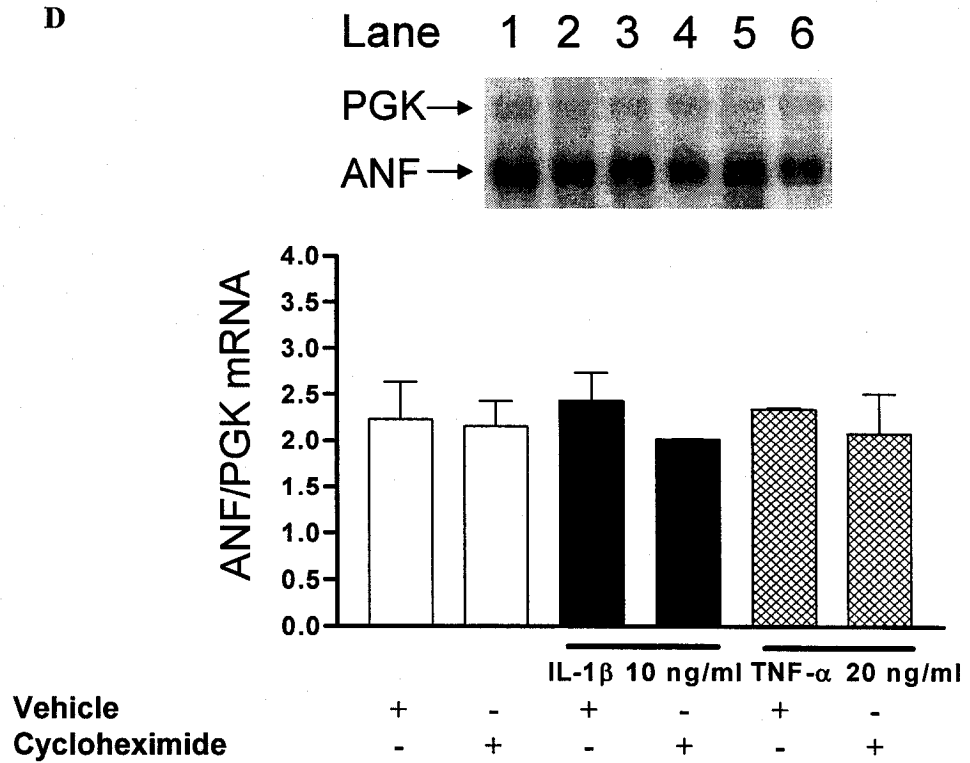


Figures 5C and 5D

C



D

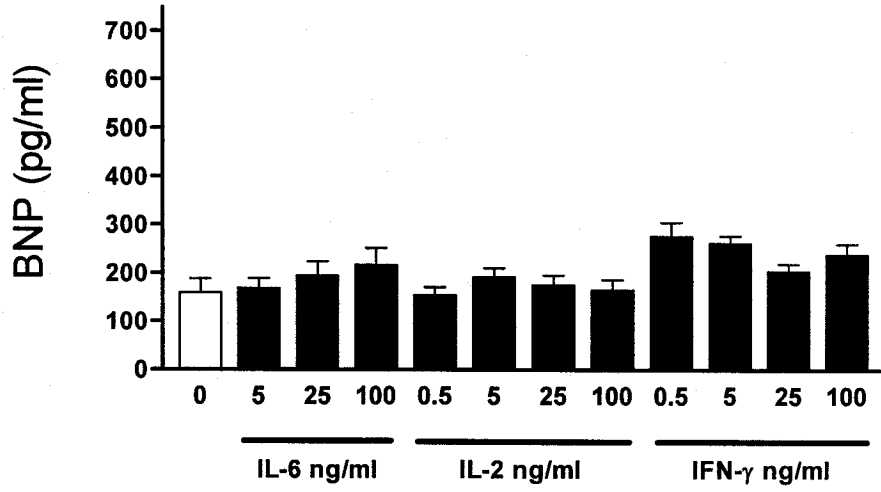


**Figures 6A and 6B:**

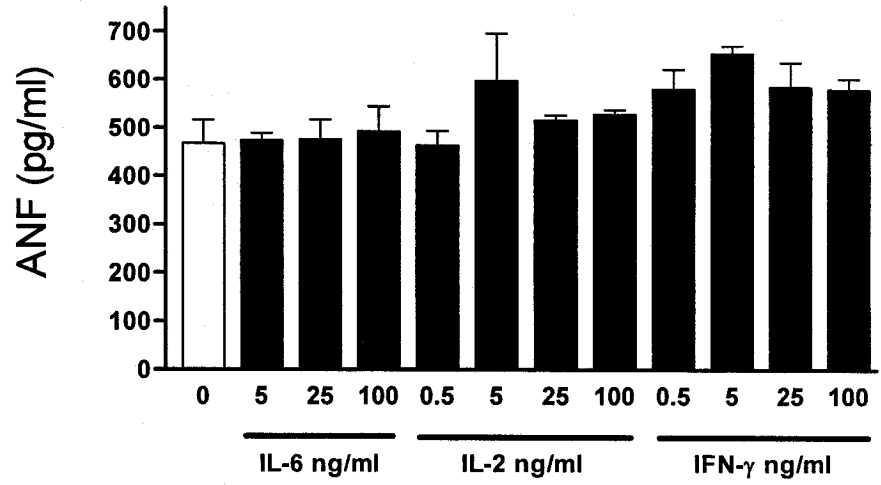
Natriuretic peptide secretion into medium by primary neonatal ventricular cardiocyte cultures stimulated by IL-6, IL-2 or IFN- $\gamma$  for 48 hours as measured by radioimmunoassay. (A) BNP secretion by IL-6 (5, 25 and 100 ng/ml) or IL-2 (0.5, 5, 25 and 100 ng/ml) or IFN- $\gamma$  (0.5, 5, 25 and 100 ng/ml). (B) ANF secretion by IL-6 (5, 25 and 100 ng/ml) or IL-2 (0.5, 5, 25 and 100 ng/ml) or IFN- $\gamma$  (0.5, 5, 25 and 100 ng/ml). Data are presented as means  $\pm$  SEM. (Statistical analysis by ANOVA). n=3 for all groups in triplicate.

Figures 6A and 6B

A



B

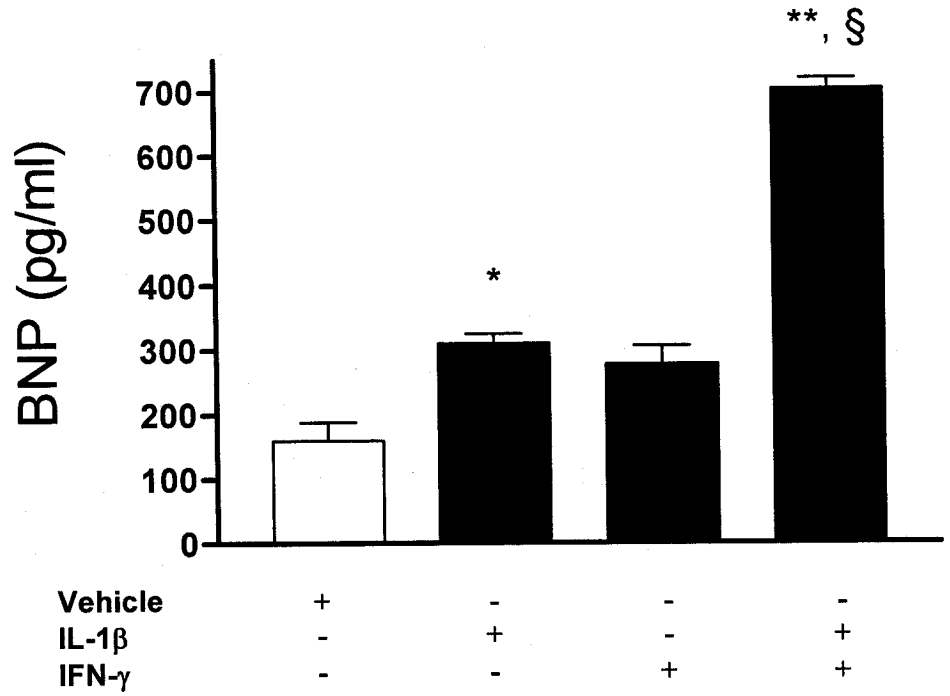


**Figures 7A and 7B:**

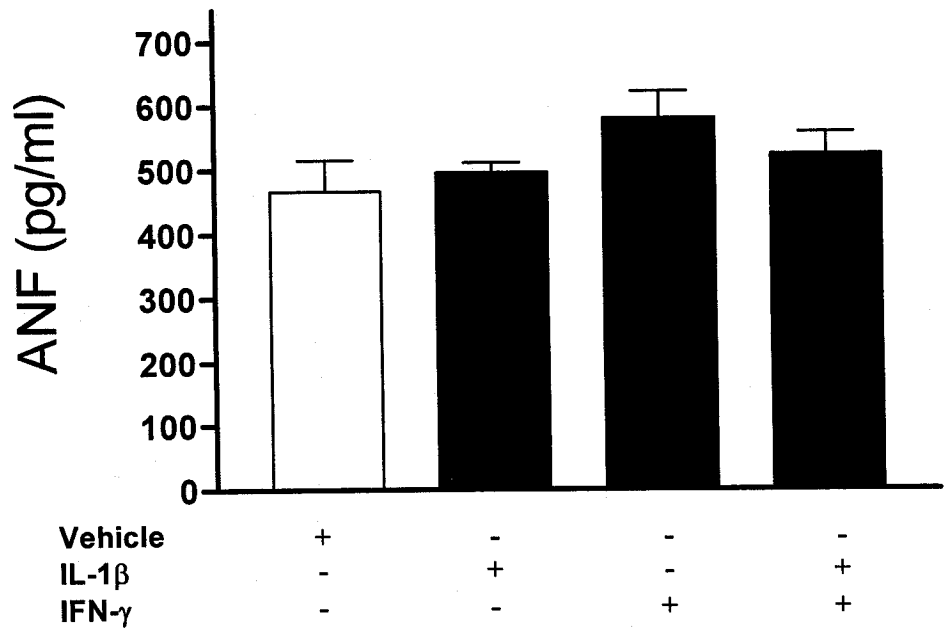
Natriuretic peptide secretion into medium by primary neonatal ventricular cardiocyte cultures stimulated by IL-1 $\beta$  and IFN- $\gamma$  in combination for 48 hours as measured by radioimmunoassay. (A) BNP secretion by IL-1 $\beta$  (5 ng/ml), IFN- $\gamma$  (0.5 ng/ml), or by IL-1 $\beta$  (5 ng/ml) + IFN- $\gamma$  (0.5 ng/ml). (B) ANF secretion by IL-1 $\beta$  (5 ng/ml), IFN- $\gamma$  (0.5 ng/ml), or by IL-1 $\beta$  (5 ng/ml) + IFN- $\gamma$  (0.5 ng/ml). Data are presented as means  $\pm$  SEM. \*, p<0.05; \*\*, p<0.01 vs. control; §, p<0.001 vs. IL-1 $\beta$  group (Statistical analysis by ANOVA). n=3 for all groups in triplicate.

Figures 7A and 7B

A



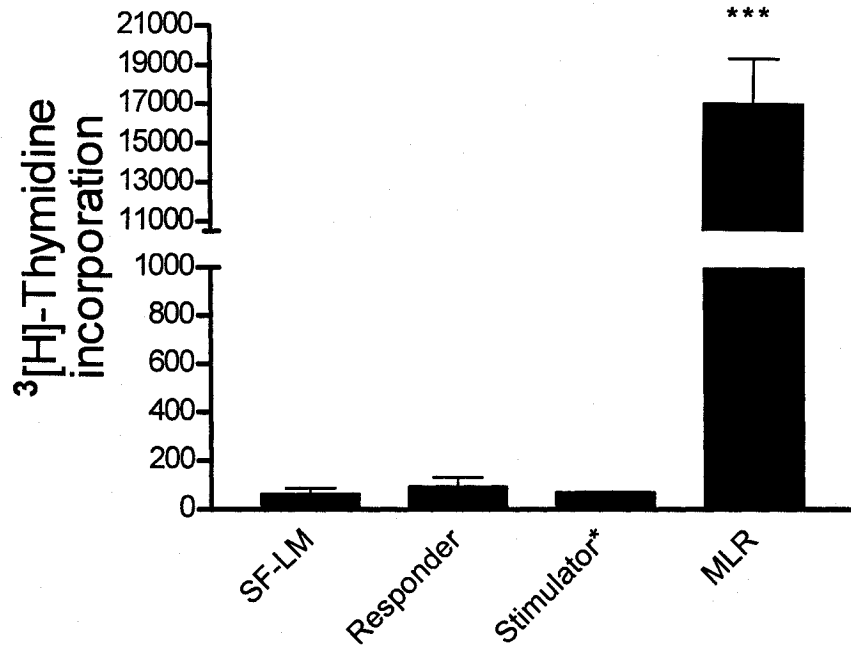
B



**Figure 8:**

<sup>3</sup>[H]-thymidine incorporation by allo-activated T-lymphocyte cultures after 4 days. A 5 day MLR was performed as outlined in the Materials and Methods section. On day 4 T-lymphocytes were pulse labeled with 1  $\mu$ Ci <sup>3</sup>[H]-thymidine and then harvested on day 5. SF-LM, serum-free lymphocyte medium; responder, responder lymphocytes alone; stimulator,  $\gamma$ -irradiated T-lymphocytes alone; MLR, serum-free mixed lymphocyte reaction of responder and stimulator lymphocytes. Data are presented as means  $\pm$  SEM. \*\*\*,  $p < 0.001$  vs. SF-LM, responder and stimulator control groups. (Statistical analysis by ANOVA).  $n=3$  for all groups in triplicate.

Figure 8

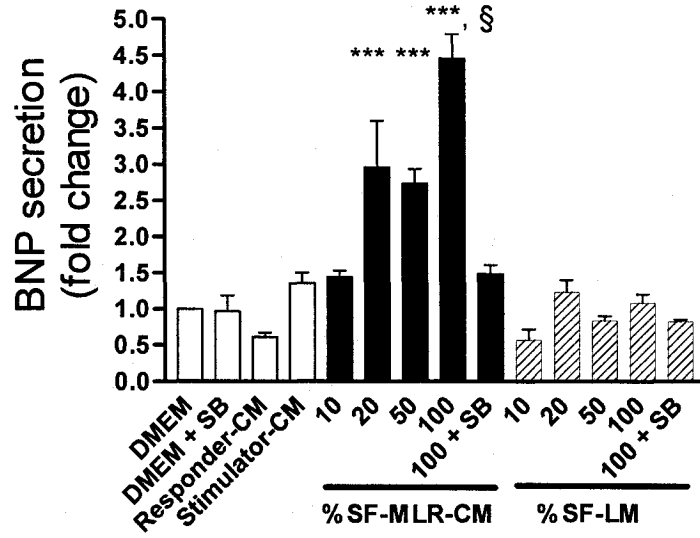


**Figures 9A and 9B:**

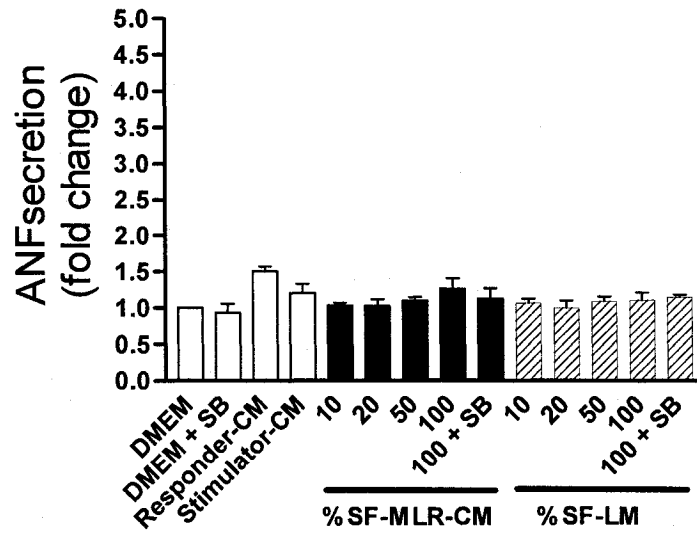
Natriuretic peptide secretion into medium by primary neonatal ventricular cardiocyte cultures stimulated by the serum-free mixed lymphocyte reaction condition medium, SF-MLR-CM after 48 hours. Supernatants from a 5 day MLR was harvested as outlined in the Materials and Methods section. In experiments using inhibitors, cultured cardiocytes were pre-incubated with SB203580 (SB) (1  $\mu$ M) or vehicle for 1 hour prior to stimulation with conditioned medium (or control) for a 48 hour duration as indicated. Data is expressed relative to DMEM control values arbitrarily set at 1.0. (A) BNP secretion by basal cardiocyte DMEM culture medium; responder-CM, responder cell conditioned medium; stimulator-CM, stimulator cell conditioned medium; SF-MLR-CM, serum-free MLR conditioned medium in 10, 20, 50 and 100% proportions; SF-LM, serum-free lymphocyte medium. (B) ANF secretion by basal cardiocyte DMEM culture medium; responder-CM, responder cell conditioned medium; stimulator-CM, stimulator cell conditioned medium; SF-MLR-CM, serum-free MLR conditioned medium in 10, 20, 50 and 100% proportions; SF-LM, serum-free lymphocyte medium. Data are presented as means  $\pm$  SEM. \*\*\*,  $p < 0.001$  vs. SF-LM, responder-CM and stimulator-CM control groups and 10% SF-MLR-CM; §,  $p < 0.001$  vs. 20% SF-MLR-CM and 50% SF-MLR-CM. (Statistical analysis by ANOVA).  $n=4$  for all groups in triplicate.

Figures 9A and 9B

A



B

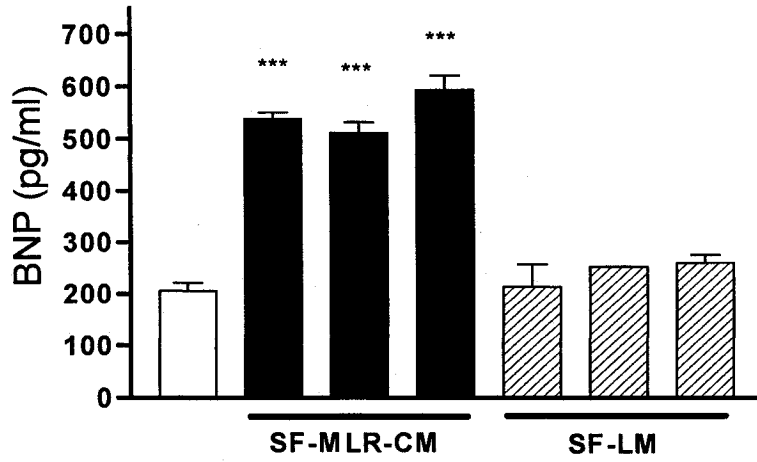


**Figures 10A and 10B:**

Natriuretic peptide secretion into medium by primary neonatal ventricular cardiocyte cultures pretreated for 1 hour with inhibitors to IL-1 $\beta$  and TNF- $\alpha$  receptors followed by stimulation with SF-MLR-CM or SF-LM as indicated for 48 hours. Cardiocytes were pretreated with IL-1RA (1  $\mu$ g/ml), sTNF-RI/RII (1  $\mu$ g/ml) or vehicle 1 hour prior to stimulation with SF-MLR-CM in 100% proportions or SF-LM control. (A) BNP secretion by SF-MLR-CM without and with IL-1RA or sTNF-RI/RII. (B) ANF secretion by SF-MLR-CM without and with IL-1RA or TNF-R I/II. Data are presented as means  $\pm$  SEM. \*\*,  $p < 0.01$  vs. control; (Statistical analysis by ANOVA).  $n=3$  for all groups in duplicate.

Figures 10A and 10B

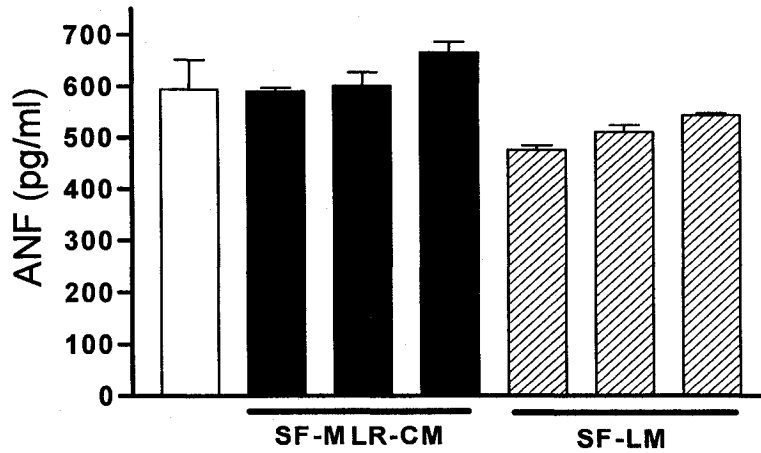
A



Vehicle  
sTNF-R1/RII  
IL-1RA

|   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|
| + | + | - | - | + | - | - |
| - | - | + | - | - | + | - |
| - | - | - | + | - | - | + |

B



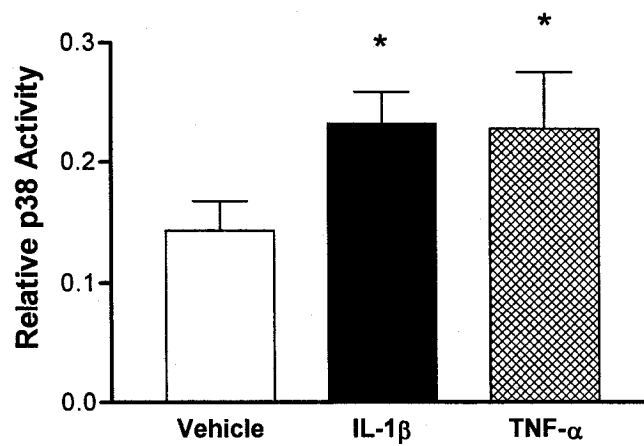
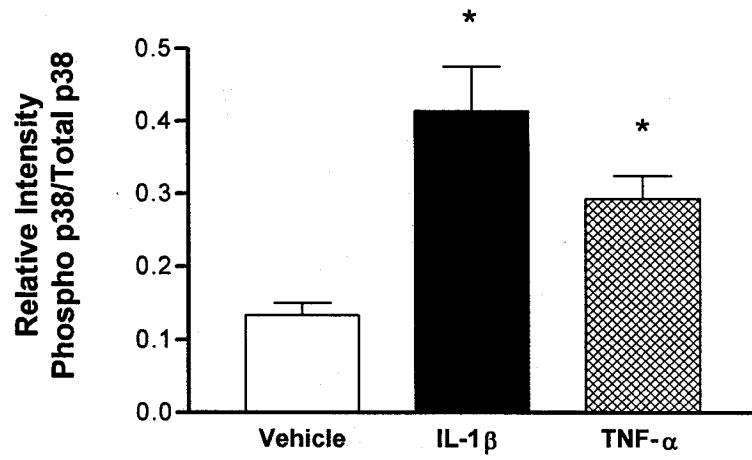
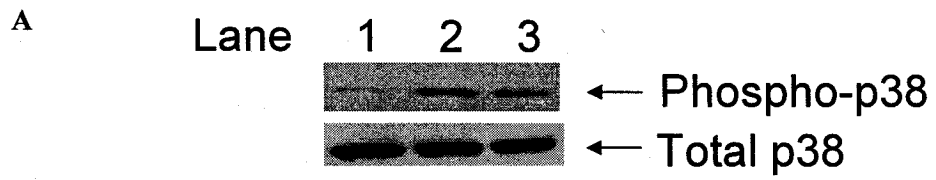
Vehicle  
sTNF-R1/RII  
IL-1RA

|   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|
| + | + | - | - | + | - | - |
| - | - | + | - | - | + | - |
| - | - | - | + | - | - | + |

**Figures 11A and 11B:**

Phosphorylation of p38 MAP kinase and upregulation p38 MAP kinase activity by IL-1 $\beta$  or TNF- $\alpha$  in primary neonatal ventricular cardiocyte cultures. (A) Representative Western blot and the densitometric quantification for relative abundance of phosphorylated p38. Lane 1, vehicle; lane 2, IL-1 $\beta$  (10 ng/ml); lane 3, TNF- $\alpha$  (20 ng/ml). Upper bands identify phosphorylated p38 by a phosphorylation specific p38 antibody (Thr180/Tyr182). The lower band identifies total p38 antibody using a non-phosphorylation specific p38 antibody. (B) Representative Western blot and the densitometric quantification for phosphorylated ATF-2 (Thr71) fusion protein using cell extracts immunoprecipitated by an immobilized monoclonal antibody to activated p38 MAP kinase (Thr180/Tyr182). Total ATF-2 was obtained using a non-phosphorylation specific polyclonal antibody. Data are presented as means  $\pm$  SEM. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  vs. control. (Statistical analysis by ANOVA).  $n = 3$  for all groups in triplicate

Figures 11A and 11B

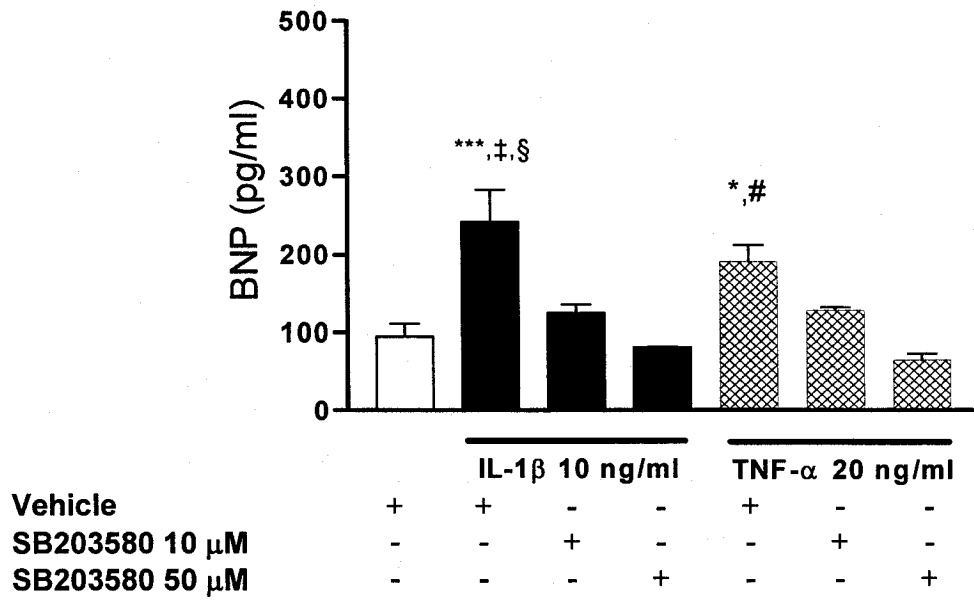


**Figures 12A and 12B:**

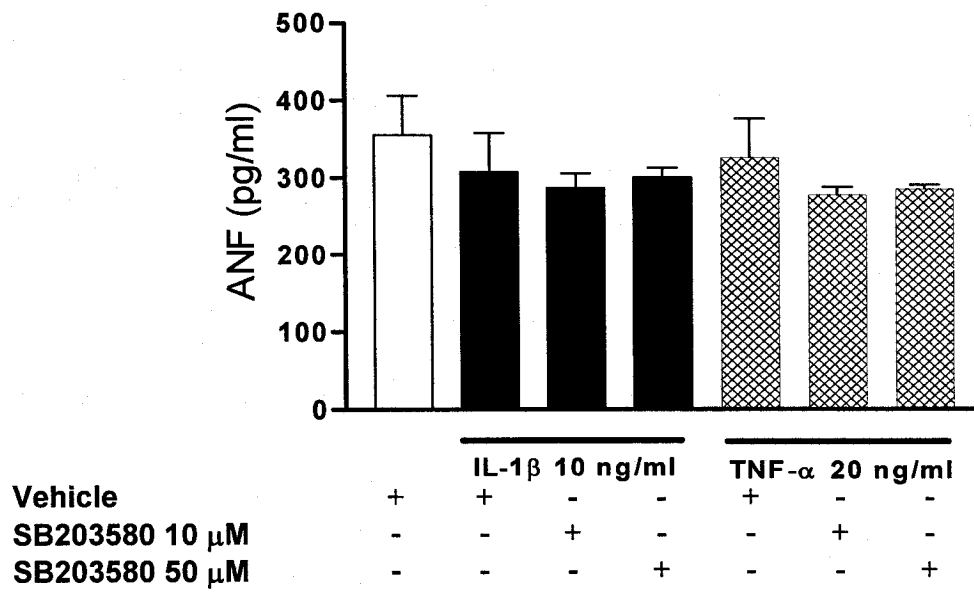
Natriuretic peptide secretion into medium by primary neonatal ventricular cardiocyte cultures pretreated for 1 hour with SB203580 followed by stimulation with either IL-1 $\beta$  or TNF- $\alpha$  as indicated for 48 hours. Cardiocytes were pretreated with SB203580 or vehicle 1 hour prior to stimulation with cytokines. (A) BNP secretion by IL-1 $\beta$  (10 ng/ml) or TNF- $\alpha$  (20 ng/ml) without and with SB203580 (10 and 50  $\mu$ M). (B) ANF secretion by IL-1 $\beta$  (10 ng/ml) or TNF- $\alpha$  (20 ng/ml) without and with SB203580 (10 and 50  $\mu$ M). Data are presented as means  $\pm$  SEM. \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$  vs. control; ‡,  $p < 0.01$  vs. IL-1 $\beta$  + 10  $\mu$ M SB203580; §,  $p < 0.001$  vs. IL-1 $\beta$  + 50  $\mu$ M SB203580; #,  $p < 0.01$  vs. TNF- $\alpha$  + 50  $\mu$ M SB203580. (Statistical analysis by ANOVA).  $n = 4$  for all groups in triplicate.

Figures 12A and 12B

A



B

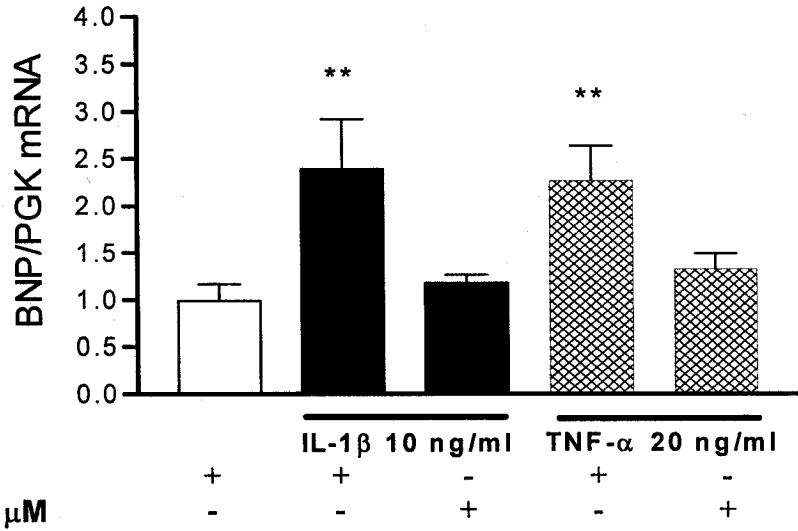
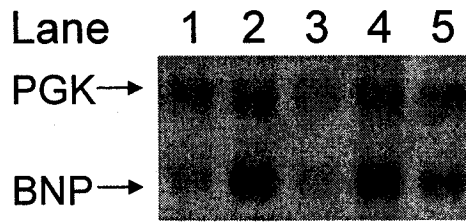


**Figures 13A and 13 B:**

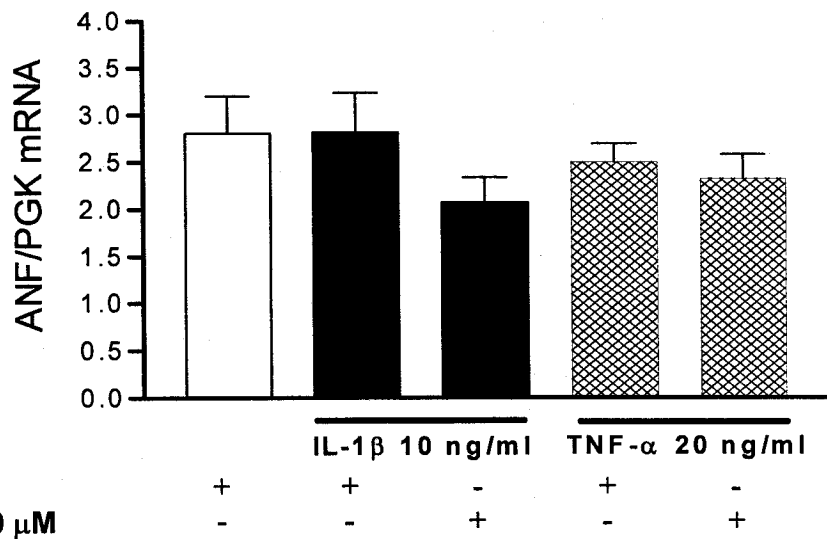
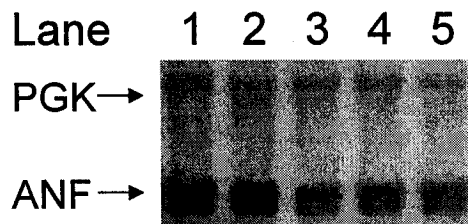
Representative Northern blot and densitometric analysis of ANF and BNP mRNA levels in primary neonatal ventricular cardiocyte cultures pretreated with SB203580 followed by stimulation with IL-1 $\beta$  or TNF- $\alpha$  for 48 hours. Cardiocytes were pretreated with SB203580 or vehicle 1 hour prior to stimulation with cytokines. Signals were normalized to PGK mRNA. (A) BNP/PGK mRNA in response to IL-1 $\beta$  (10 ng/ml) or IL-1 $\beta$  + 50  $\mu$ M SB203580 and TNF- $\alpha$  (20ng/ml) or TNF- $\alpha$  + 50  $\mu$ M SB203580 for 48 hours. (B) ANF/PGK mRNA in response to IL-1 $\beta$  (10 ng/ml) or IL-1 $\beta$  + 50  $\mu$ M SB203580 and TNF- $\alpha$  (20ng/ml) or TNF- $\alpha$  + 50  $\mu$ M SB203580 for 48 hours. Data are presented as means  $\pm$  SEM. \*\*,  $p < 0.01$  vs. control (Statistical analysis by ANOVA).  $n = 4$  for all groups in triplicate.

Figures 13A and 13B

A



B

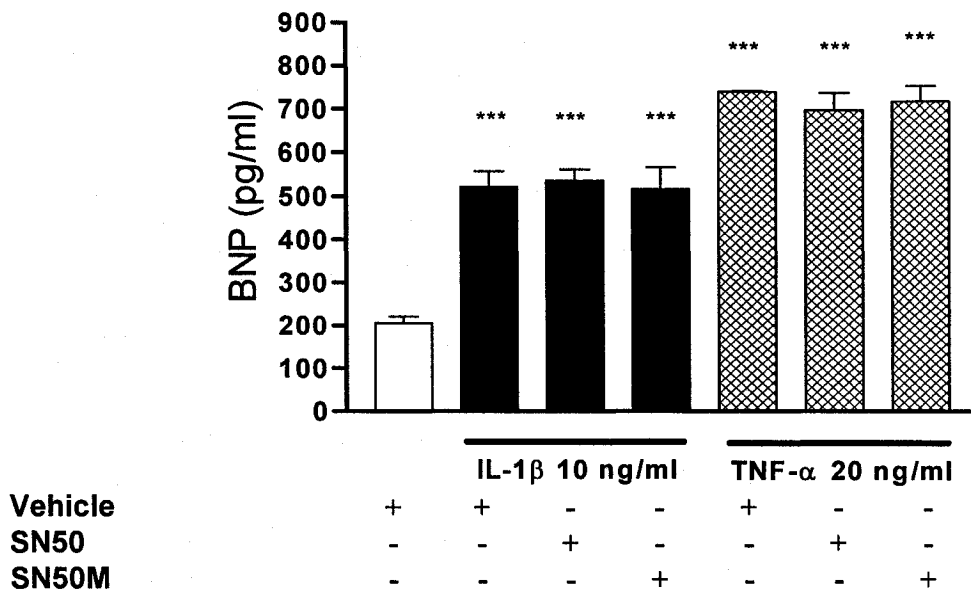


**Figures 14A and 14B:**

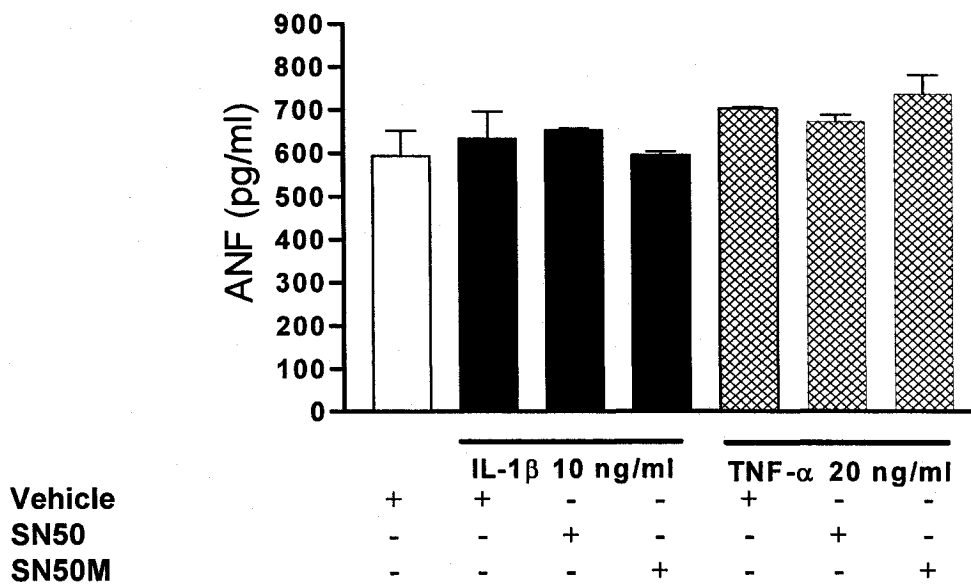
Natriuretic peptide secretion into medium by primary neonatal ventricular cardiocyte cultures pretreated for 1 hour with an inhibitor to NF- $\kappa$ B followed by stimulation with either IL-1 $\beta$  or TNF- $\alpha$  as indicated for 48 hours. Cardiocytes were pretreated with SN50 (1  $\mu$ g/ml), SN50M (1  $\mu$ g/ml) or vehicle 1 hour prior to stimulation with cytokines. (A) BNP secretion by IL-1 $\beta$  (10 ng/ml) or TNF- $\alpha$  (20 ng/ml) with SN50, SN50M or vehicle. (B) ANF secretion by IL-1 $\beta$  (10 ng/ml) or TNF- $\alpha$  (20 ng/ml) with SN50, SN50M or vehicle. Data are presented as means  $\pm$  SEM. \*\*,  $p < 0.01$  vs. control. (Statistical analysis by ANOVA).  $n = 3$  for all groups in duplicate.

Figures 14A and 14B

A



B



**Figure 15:**

$\beta$ -galactosidase histochemical staining of 2 day-old cultured neonatal rat ventricular cardiocytes transfected with a pSV- $\beta$ -galactosidase expression plasmid. Image is magnified 100X.

**Figure 15**

**Figure 16:**

Activation of a transfected -2.2 kbp rat BNP promoter by IL-1 $\beta$  and TNF- $\alpha$ . Cardiocytes were transfected with a -2.2 kbp rat BNP promoter coupled with a luciferase gene (1  $\mu$ g) or control pSV  $\beta$ -galactosidase expression plasmid (0.5  $\mu$ g) using Fugene 6<sup>®</sup> reagent followed by treatment with IL-1 $\beta$ , TNF- $\alpha$  or ET-1 with or without various inhibitors to the p38 (SB203580) or p44/p42 ERK (U0126) MAP kinase pathways. Detection was performed using the Dual Light<sup>®</sup> chemiluminescence assay system (Applied Biosystems). Promoter activation is expressed as luciferase activity normalized to  $\beta$ -galactosidase activity relative to the fold activation over the non-stimulated control set at 1.0. Data are presented as means  $\pm$  SEM. \*, p<0.5; \*\*, p<0.01; \*\*\*, p<0.001 vs. control; †, p<0.05 vs. U0126 alone; §, p<0.05 vs. SB203580 alone. (Statistical analysis by ANOVA). n=4 for all groups in duplicate.



## DISCUSSION

ANF and BNP are continuously released from the heart and are potent modulators of vascular tone, fluid and electrolyte balance and cardiovascular growth. As a rule, under pathophysiological conditions such as chronic hypertension or chronic congestive heart failure, the expression and secretion of both hormones are significantly augmented. An exception to this rule is seen in acute cardiac allograft rejection episodes when BNP but not ANF plasma levels are increased (Masters et al., 1999). Successful treatment of rejection with anti-T-lymphocyte therapy decreases BNP plasma levels to pre-rejection levels (Masters et al., 1999). These results suggest that increased pro-inflammatory cytokine production seen during the acute inflammatory response of rejection might be responsible for causing the rapid and selective rise in plasma BNP. The molecular mechanisms that play a role in the selective upregulation of BNP gene expression have never been examined.

The present study examined the effects of cytokines IL-1 $\beta$ , IL-2, IL-6, TNF- $\alpha$ , and IFN- $\gamma$  on natriuretic peptide gene expression and secretion from isolated ventricular cardiocyte cell cultures. The choice of cytokines was based on the pro-inflammatory and immunomodulatory cytokines that are most likely to be present at the site of allograft rejection. Cytokines in combination were also tested for the ability to cooperatively stimulate the secretion of natriuretic peptides. In addition, we examined the secondary signaling pathways used by pro-inflammatory cytokines to selectively increase BNP promoter activity, mRNA, protein expression. Lastly, conditioned medium from allogeneically activated mixed T-lymphocytes was added to

the cultured neonatal rat ventricular cardiocytes to test for the ability to modulate cardiac natriuretic peptide gene expression.

The results of this study reveal that the regulation of BNP expression by pro-inflammatory cytokines is markedly different than for ANF. Only BNP mRNA and protein expression increased significantly after stimulation with pro-inflammatory cytokines and conditioned media from allo-activated mixed T-lymphocytes.

### **IL-1 $\beta$ increased BNP secretion and mRNA**

In the present work, pro-inflammatory cytokine IL-1 $\beta$  at concentrations ranging from 0.5 to 10 ng/ml dose-dependently increased BNP secretion after 24 and 48 hours. After 48 hours of incubation, IL-1 $\beta$  also increased BNP mRNA transcript abundance. In contrast, neither ANF secretion nor mRNA abundance was significantly altered by any concentration of IL-1 $\beta$ . Our results with IL-1 $\beta$  are generally in line with those from other investigations. Harada *et al.* also showed that IL-1 $\beta$  (1 ng/ml) significantly stimulated BNP but not ANF secretion from cultured NRVCs after 48 hours (Harada *et al.*, 1999). Unfortunately, this study by Harada *et al.* failed to examine the changes in levels ANF and BNP mRNA after treatment with IL-1 $\beta$  nor did they examine any of the secondary signaling processes responsible for this phenomenon. Furthermore, a study by Horio *et al.* also did not observe changes in ANF secretion after 24 hours or 48 hours of IL-1 $\beta$  treatment (Horio *et al.*, 1998).

The concentration of cytokines (IL-1 $\beta$ , TNF- $\alpha$ , IL-2, IL-6 and IFN- $\gamma$ ) used in our experiments was chosen to reflect the presence of elevated serum levels seen during allograft rejection which are typically between 10 to 300 pg/ml (Azzawi *et al.*,

1996), acute myocardial infarction (Blum et al., 1994; Guillen et al., 1995) and heart failure (Testa et al., 1996; Nozaki et al., 1997). Given that serum levels of cytokines do not accurately reflect local amounts, it is conceivable that cytokine concentrations seen at the local level are very high (Krown et al., 1996). In further support of this notion, it has been demonstrated that the heart can be induced to synthesize cytokines such as TNF- $\alpha$  (Kapadia et al., 1995). It is noteworthy to mention that similar concentrations have been used to examine the effects of these cytokines on cardiocyte gene expression (Thaik et al., 1995; Patten et al., 1996; Sharma et al., 1996; Horio et al., 1998; Harada et al., 1999).

IL-1 $\beta$  is a 17-kDa protein mainly synthesized and secreted by the lymphocytes and macrophages and participates in the inflammatory response to foreign antigens. IL-1 levels are increased during inflammation and allograft rejection. There are three members of the interleukin 1 family, IL-1 $\alpha$ , IL-1 $\beta$  and the soluble IL-1 receptor antagonist (IL-1RA) which are structurally related, but IL-1 $\alpha$  lacks a leader peptide and therefore is not found in appreciable amounts in normal plasma (Dinarello, 1997). All bind with similar affinity to the two types of interleukin-1 receptors (IL-1R) (Dinarello, 1997). IL-1RI is a 85-kDa glycoprotein containing 3 Ig-like loop domains and belongs to the immunoglobulin supergene family (Sims and Dower, 1994). IL-1RII is a 68-kDa glycoprotein, but unlike IL-1RI, IL-1RII can be shed from the membrane, does not transduce IL-1 signaling and therefore is believed to act as an IL-1 sink (Sims et al., 1994).

It remains unclear what the immediate downstream events are after IL-1RI activation, but there is an increase in GTPase activity that is pertussis toxin sensitive

consistent with a hypothesis that G<sub>i</sub>-proteins may be partly involved (O'Neill et al., 1990). Protein kinase A (Chedid and Mizel, 1990) and protein kinase C (Zucali et al., 1990) activation have also been observed, but their involvement still remains controversial. Lipid-mediated signaling such as the sphingomyelin and ceramide (Kolesnick and Golde, 1994) and the prostaglandin E<sub>2</sub> and phospholipase A<sub>2</sub> (Angel et al., 1994) pathways are also believed to be downstream of IL-1R activation. The ambiguity in the identity of the precise pathways downstream of IL-1R may be mainly due to the conflicting data collected from differentiated versus undifferentiated cell systems and the variation in culture conditions. Lastly, IL-1 signaling has also been shown to activate the three mammalian mitogen-activated protein kinases, p44/42 ERK, p38 and p54/46 c-Jun N-terminal Kinase (JNK) (Saklatvala et al., 1999). The latter two kinases are commonly referred to as the stress-activated MAP kinases (SAPKs) (Force and Bonventre, 1998) as the SAPKs are also activated by multiple stress stimuli such as ultraviolet light, shear and osmotic stress (Bogoyevitch, 2000; Kyriakis and Avruch, 2001).

In addition to being a primary mediator of inflammation, IL-1 $\beta$  also acts on many cell types outside the immune system (Bankers-Fulbright et al., 1996). In the heart, IL-1 $\beta$  can modulate myocardial function by regulating the expression of cardiac genes that may be either detrimental or beneficial to cardiac function. IL-1 $\beta$  has been shown to decrease collagen synthesis and increase matrix metalloproteinases (MMP) to promote myocardial remodeling (Siwik et al., 2000). IL-1 $\beta$  can depress cardiac contractility (Hosenpud et al., 1989), in part, via upregulating iNOS expression (inducible nitric oxide synthase) and nitric oxide production (Balligand et al., 1994; LaPointe and Isenovic, 1999). However, the influence of IL-1 $\beta$  on cardiac

contractility remains controversial since other studies have shown that IL-1 $\beta$  does not alter basal cardiac contractility (Gulick et al., 1989; McTiernan et al., 1997). On the other hand, IL-1 $\beta$  can increase cardiac contractility and responsiveness by decreasing expression of phospholamban, a key regulatory protein of sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA) (McTiernan et al., 1997). It is known that IL-1 $\beta$  can also have a profound influence in the upregulation of genes that may be cardioprotective in times of stress, in particular the upregulation of superoxide dismutase and heat shock proteins in myocardial ischemia reperfusion injury (Maulik et al., 1993; Nogue et al., 1995). IL-1 $\beta$  can also be a potent stimulator of cardiocyte protein synthesis and inhibitor of fibroblast proliferation (Palmer et al., 1995).

Our study, and that done by Harada *et al.* (Harada et al., 1999) and Horio *et al.* (Horio et al., 1998) are in disagreement with that of Thaik *et al.* who reported that IL-1 $\beta$  treatment can lead to the upregulation of ANF mRNA (Thaik et al., 1995). The reason for this discrepancy is not clear, but it may be related to differences in purity of the isolated cardiocytes, the culture conditions and amounts of cytokine used to stimulate the NRVCs. For instance, Harada *et al.* used co-cultures of cardiocytes and non-cardiocytes (primarily fibroblast) and discovered that IL-1 $\beta$  stimulated both ANF and BNP mRNA and secretion. These authors hypothesized that the release of uncharacterized hypertrophic growth factors secreted by the fibroblast were necessary for this effect (Harada et al., 1999). At present, there is no general consensus that IL-1 $\beta$  can directly induce cardiac hypertrophy as typically seen with myocardial stretch, vasoactive peptides such as ET-1, ANG-II or  $\alpha$ -adrenergic agonists. A transgenic mouse model overexpressing human IL-1 $\alpha$  presents with what is closely associated with cardiac hypertrophy such as increased left ventricular weight/body weight ratio

and upregulation of ANF and  $\beta$ -MHC expression (Isoda et al., 2001). However, this study may have serious limitations since transgene overexpression was not targeted to the heart with a myocardial  $\alpha$ -MHC promoter, rather a systemic upregulation of IL-1 was produced by using a cytomegalovirus enhancer and chicken  $\beta$ -actin promoter. Also, these animals suffered from very poor growth rates further complicating the interpretation of the results. From *in vitro* studies, it has been demonstrated that the cell growth induced by IL-1 $\beta$  is noticeably different than that induced by the  $\alpha$ -adrenergic agonist, PE. Whereas PE causes an increase in both length and width of the cardiocyte, IL-1 $\beta$  increases only cell length. IL-1 $\beta$ -mediated increase in cell length can be accompanied by an increase in peri-nuclear localization of ANF protein (Ng et al., 2001) and protein synthesis in general (Harding et al., 1995). Unfortunately effects induced by IL-1 $\beta$  on ANF or BNP secretion and mRNA levels were not examined by Ng *et al.* (Ng et al., 2001). In further support of the notion that IL-1 $\beta$  may not be a typical hypertrophic agonist, co-incubation of IL-1 $\beta$  with PE depresses PE-mediated upregulation of hypertrophic marker genes like skeletal  $\alpha$ -actin,  $\beta$ -myosin heavy chain ( $\beta$ -MHC) mRNA (Patten et al., 1996) and BNP secretion (Harding et al., 1995). This seemingly paradoxical mechanism of negative regulation of PE-induced cardiac hypertrophy by IL-1 $\beta$  has not been elucidated but may involve activation of transcriptional repressors like the YY1 transcription factor (Patten et al., 2000).

In summary, IL-1 $\beta$  is not a typical hypertrophic agonist that increases the expression of both ANF and BNP hypertrophic marker genes in NRVCs. Our study demonstrates that the effects of IL-1 $\beta$  on natriuretic peptide gene expression are quite

selective, such that only BNP secretion and mRNA, but not ANF is significantly upregulated by IL-1 $\beta$ .

### **TNF- $\alpha$ increases BNP secretion and mRNA**

In the present work a novel observation was made that TNF- $\alpha$  dose-dependently and selectively stimulated BNP mRNA and BNP secretion from cardiocyte cultures. To our knowledge, the effect of TNF- $\alpha$  on BNP mRNA and secretion from neonatal rat ventricular cardiocyte cultures has not been previously reported. Similar to IL-1 $\beta$ , TNF- $\alpha$  also did not upregulate ANF secretion nor did it increase steady state BNP mRNA levels. Our results are in line with Horio *et al.* who also reported that TNF- $\alpha$  (20 ng/ml) did not alter ANF secretion and mRNA from cultured neonatal rat ventricular cardiocytes. BNP is not the sole gene to be upregulated by TNF- $\alpha$  in the cardiocyte as it is believed that TNF- $\alpha$  can modulate the expression of many cardiac genes that are involved in cardiac remodeling and heart failure (Mann, 2001). TNF- $\alpha$  has been shown to induce a modest form of cardiac hypertrophy (Yokoyama *et al.*, 1997) and other groups have reported increased ventricular re-expression of natriuretic peptides is seen in transgenic mice with cardiac-specific TNF- $\alpha$  overexpression (Kubota *et al.*, 1997). These transgenic mice develop dilated cardiomyopathy and capitulate the phenotype associated with chronic heart failure with ventricular hypertrophy (Kubota *et al.*, 1997).

The pro-inflammatory cytokine TNF- $\alpha$  is a 17-kDa protein produced mainly by activated macrophages. It is also known that lymphocytes, mast cells, endothelial cells, fibroblasts, smooth muscle cells (Azzawi and Hasleton, 1999) and in special circumstances, cardiocytes may also synthesize and secrete TNF- $\alpha$  (Kapadia *et al.*,

1995; Ballard-Croft et al., 2001). TNF- $\alpha$  can exist in a membrane-bound form or be secreted as a non-covalently linked homotrimer. In the adult heart, TNF- $\alpha$  binds to either the type I (TNFRI/p55) or to the lesser-known type II (TNFRII/p75) homotrimeric receptor (Torre-Amione et al., 1995). TNFRI signal transduction begins with activation of TNFR-associated death domain (TRADD) protein. If TRADD couples with Fas-associated death domain (FADD), the result is recruitment of caspases and cellular apoptosis (Haunstetter and Izumo, 1998). On the other hand, if TRADD associates with TNFR-associated factor 2 (TRAF2), this will lead to nuclear factor- $\kappa$ B (NF- $\kappa$ B)-dependent increased expression of genes involved in protection against cardiocyte apoptosis (Natoli et al., 1998; Sack et al., 2000; Kurrelmeyer et al., 2000) or NF- $\kappa$ B-independent events that culminate in the activation of p38 and JNK SAPKs (Roulston et al., 1998; Kyriakis, 1999).

In summary, the stimulation of NRVCs with the pro-inflammatory cytokine TNF- $\alpha$  selectively and significantly upregulated BNP mRNA and secretion, whereas, the same concentrations of TNF- $\alpha$  did not alter ANF mRNA or secretion.

### **The role of p38 MAP Kinase and NF- $\kappa$ B in mediating IL-1 $\beta$ - and TNF- $\alpha$ - increase in BNP secretion and gene expression**

The pro-inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  share many similar downstream effects. The precise downstream secondary signaling pathways activated by the pro-inflammatory cytokine IL-1 $\beta$  to alter gene expression are not entirely known (Dinarello, 1997), but better characterized are the pathways activated by TNF- $\alpha$  (Krown et al., 1996; Ferrari, 1998; Natoli et al., 1998; Condorelli et al., 2002). A common family of enzymes activated by IL-1 $\beta$  and TNF- $\alpha$  are the two SAPKs, p38

and JNK. Recent experiments suggest that the SAPKs may be responsible for IL-1 $\beta$  and TNF- $\alpha$ -mediated upregulated expression of many genes in the myocardium (Bogoyevitch, 2000). LaPointe *et al.* have shown that p38 and p44/42 ERK MAP kinase pathways mediate IL-1 $\beta$  upregulation of cardiocyte iNOS expression (LaPointe and Isenovic, 1999). IL-1 $\beta$  also increases the expression of group Ila phospholipase A<sub>2</sub> via activation of p38 MAP kinase (Degousee *et al.*, 2001). Ng *et al.* have shown that IL-1 $\beta$  can increase cardiocyte length and peri-nuclear ANF expression and that inhibitors of p38 MAP kinase attenuate this increase in intracellular ANF expression (Ng *et al.*, 2001). In their study, Ng *et al.* did not examine ANF or BNP secretion or mRNA levels (Ng *et al.*, 2001). Overexpression of p38 and JNK *in vitro* (Zechner *et al.*, 1997; Wang *et al.*, 1998) and *in vivo* (Choukroun *et al.*, 1998; Choukroun *et al.*, 1999; Liao *et al.*, 2001) has revealed that the SAPKs can regulate the expression of the cardiac hypertrophic marker genes,  $\beta$ -MHC, skeletal  $\alpha$ -actin and the natriuretic peptides.

We found that within 15 minutes of stimulation with IL-1 $\beta$  and TNF- $\alpha$ , there was a significant increase in phosphorylated p38 abundance as determined by western blot using polyclonal antibodies to phosphorylated p38 MAP kinase (Thr180/Tyr182). To assess whether p38 activity was also increased, an *in vitro* kinase assay was performed using an ATF-2 fusion protein as the p38 substrate. Indeed, within 15 minutes, ATF-2 phosphorylation (Thr71) was also significantly increased in the IL-1 $\beta$  and TNF- $\alpha$  treatment groups.

Furthermore, to examine if p38 was playing a major role in transducing cytokine-specific upregulation of BNP gene expression and secretion, we pre-

incubated NRVCs with an inhibitor of p38 MAP kinase, SB203580, 1-hour prior to stimulation with IL-1 $\beta$  or TNF- $\alpha$ . SB203580 is a class of pyridinyl imidazoles that inhibit the activity of p38 MAP kinase by competing for ATP binding (Kumar et al., 1999; Lee et al., 1999). The functional significance of p38 inhibition was the complete abolition of IL-1 $\beta$  or TNF- $\alpha$ -mediated upregulation of BNP steady state mRNA levels and BNP. SB203580 at the concentrations used in this study (1  $\mu$ M up to 50  $\mu$ M) are specific to p38 as it has been demonstrated that concentrations up to 100  $\mu$ M do not inhibit the activities of JNK or p44/42 ERK MAP kinases (Cuenda et al., 1995). SB203580 inhibits p38 MAP kinase with an IC<sub>50</sub> of 0.6  $\mu$ M in HeLa cells (Cuenda et al., 1995). Although a recent report suggests that IL-1 $\beta$  and TNF- $\alpha$  activates the three MAP kinases, p44/42 ERK, JNK and p38 (Clerk et al., 1999), the study by Clerk *et al.* did not examine in further detail the genes in cardiocytes that were activated by these kinases. Therefore, in light of our p38 inhibition experiments, our results strongly suggest that neither ERK nor JNK pathways act in parallel with p38 to mediate IL-1 $\beta$ - and TNF- $\alpha$ -induction of BNP gene expression. The p38 MAP kinase substrate activated by these cytokines remains unknown.

NF- $\kappa$ B is a transcription factor activated by IL-1 $\beta$  and TNF- $\alpha$ . NF- $\kappa$ B is a heterodimer of p65(RelA)/p50 subunits but other combinations of the five proteins belonging to the NF- $\kappa$ B family are known (Mercurio and Manning, 1999). In quiescent cells, NF- $\kappa$ B remains cytosolic and bound to I $\kappa$ B $\alpha$  protein. NF- $\kappa$ B activation begins with phosphorylation of I $\kappa$ B $\alpha$  by I $\kappa$ B kinase (IKK). As a result, I $\kappa$ B $\alpha$  becomes polyubiquitinated and degraded, thereby permitting the translocation of NF- $\kappa$ B into the nucleus. In the heart, activated NF- $\kappa$ B may be involved in enhancing

the expression of genes associated with protection against myocardial apoptosis (Mustapha et al., 2000) and cardiac hypertrophy (Higuchi et al., 2002) after stimulation with TNF- $\alpha$  or by PE, ANG-II and ET-1 stimulation (Purcell et al., 2001). Unfortunately, the identity of the genes activated by NF- $\kappa$ B remain unknown since none these studies addressed this question. It is interesting to note that the 5'-flanking region of the human BNP promoter contains shear stress responsive elements (SSREs) that bind NF- $\kappa$ B. These SSREs elements are critical in the activation of BNP transcription in response to *in vitro* stretch (Liang and Gardner, 1999).

We attempted to determine if interference with the NF- $\kappa$ B pathway would diminish the IL-1 $\beta$  and TNF- $\alpha$ -mediated increase in BNP secretion by cardiocytes. A cell-permeable inhibitory peptide, SN50 at a concentration (25  $\mu$ g/ml) did not decrease IL-1 $\beta$ - or TNF- $\alpha$ -mediated induction of BNP secretion. SN50 contains the hydrophobic region of Kaposi fibroblast growth factor (K-FGF), which confers cell permeability and a p50 subunit nuclear localization sequence (NLS) that competes with native NF- $\kappa$ B for the recognition of the NLS by intracellular machinery. At appropriate concentrations this prevents native NF- $\kappa$ B translocation and function without cross-reactivity with other nuclear proteins (Lin et al., 1995). An inactive control peptide, SN50M, which contains a mutated form of the p50 NLS did not cause any detectable adverse effects and as expected did not alter IL-1 $\beta$ - and TNF- $\alpha$ -mediated increase in BNP secretion. These results suggest that the NF- $\kappa$ B pathway is not involved in transducing changes in BNP gene expression following IL-1 $\beta$  and TNF- $\alpha$  stimulation.

In summary, immediately following IL-1 $\beta$  and TNF- $\alpha$  stimulation, there is a significant increase in phosphorylated p38 MAP kinase abundance and kinase activity. Inhibiting the p38 MAP kinase pathway with SB203580 at concentrations known to abolish activity of p38 while not affecting other MAP kinases completely suppressed IL-1 $\beta$  and TNF- $\alpha$ -induced increase in BNP mRNA and secretion from NRVCs. The inhibition of NF- $\kappa$ B with SN50 did not appreciably inhibit IL-1 $\beta$  and TNF- $\alpha$ -mediated increases in BNP secretion and therefore this transcription factor is not responsible for the upregulation of BNP gene expression by IL-1 $\beta$  and TNF- $\alpha$ .

### **IL-1 $\beta$ and TNF- $\alpha$ increase BNP gene expression by a transcriptional mechanism**

The increase in IL-1 $\beta$ - and TNF- $\alpha$ -induced BNP mRNA abundance was sensitive to the transcriptional inhibitor actinomycin D. This suggests that an increase in transcription was the primary mechanism responsible for the increase in BNP mRNA after stimulation with IL-1 $\beta$  and TNF- $\alpha$ . To test these ideas two different approaches were used. The first one involved transiently transfecting NRVC cultures with a BNP promoter linked to a luciferase reporter gene to examine whether there was a direct relationship between IL-1 $\beta$  and the increase in BNP mRNA transcription. The second approach involved the use of protein synthesis inhibitors to examine whether post-transcriptional translation-dependent mechanisms are playing a role in enhancing levels of BNP mRNA after IL-1 $\beta$  and TNF- $\alpha$  stimulation.

In our study, we demonstrate for the first time that IL-1 $\beta$  and TNF- $\alpha$  stimulates the activation of a rat -2.2-kbp BNP promoter. The rat -2.2-kbp BNP promoter has been shown to be of sufficient length to confer both basal tissue-specific

and induced increases in BNP gene expression by hypertrophic agonists such as myocardial stretch and ET-1 (Grepin et al., 1994; Thuerlauf et al., 1994; Thuerlauf and Glembotski, 1997; Durocher et al., 1997; Pikkarainen et al., 2003a). From our experiment with inhibitors to p38 and ERK MAP kinase, it was shown that IL-1 $\beta$  and TNF- $\alpha$ -induced BNP promoter activation was dependent on p38 but independent of ERK MAP kinase activity. The precise p38 substrate(s) and DNA consensus elements in the 5' BNP promoter that may be responsible for the cytokine-mediated increase in promoter activity have not yet been characterized, however, a survey of the recent literature has revealed the presence of some candidate *cis*-acting consensus elements. IL-1 $\beta$  can upregulate a transcriptional regulator YY1 in NRVCs (Patten et al., 2000). YY1 is a 65-kDa zinc finger DNA binding protein that belongs to the human GLI-Krüppel family of nuclear proteins that is known mostly as a transcriptional repressor in many cell types (Shi et al., 1991). A direct relationship between IL-1 $\beta$ , YY1 and the BNP promoter has yet to be proven, but a recent study has demonstrated that YY1 can actually be an activator of cardiac muscle specific genes by cooperating with another transcription factor, GATA-4 to synergistically activate the -2.2 kb rBNP promoter (Bhalla et al., 2001). Unfortunately it has not been determined if YY1 is directly downstream of p38 activation, but interestingly, GATA-4 has been shown to be phosphorylatable by p38 MAP (Kerkela et al., 2002) and possibly by ERK MAP kinases (Liang et al., 2001b). Therefore, circumstantial evidence does provide a connection between pro-inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$ , p38, transcriptional co-activators YY1 and GATA and activation of the BNP promoter.

The M (muscle)-CAT consensus element found in the 5' proximal promoter of the rat (Thuerlauf and Glembotski, 1997) and human BNP genes (He and LaPointe,

1999) is another potential IL-1 $\beta$  (and TNF- $\alpha$ ) responsive *cis*-acting element. The M-CAT and the associated transcription enhancement factor (TEF) are believed to confer tissue-specific and inducible regulation of many cardiac- and skeletal-specific genes (LaPointe et al., 1996; Larkin et al., 1996). Interestingly, no M-CAT sequences have been reported to be present in either the rat or human proximal ANF promoters. He *et al.* has shown that IL-1 $\beta$  targets the M-CAT element exclusively to increase human BNP promoter activity and this activity was partially dependent on signaling by p38 MAP kinase (He and LaPointe, 1999). The GATA elements do not appear to cooperate with M-CAT elements in IL-1 $\beta$ -mediated induction of the human BNP promoter (He and LaPointe, 1999) unlike that seen in the rat BNP promoter suggesting that factors such as species diversity may be relevant in studying the regulation of the BNP promoters.

In our present study, we cannot definitively conclude that M-CAT and/or YY1 or other consensus elements such as GATA were responsible for mediating IL-1 $\beta$  and/or TNF- $\alpha$ -mediated increases in rat BNP promoter activity since these aims are beyond the scope of this project. However, since we have shown that similar to the study by He *et al.* (He and LaPointe, 1999), the IL-1 $\beta$ -mediated increase in BNP promoter activity was suppressed with p38 MAP inhibition, it is very likely that p38 signaling is required for transcription factor activation. Furthermore, the pharmacological blockade of p38 also abolished TNF- $\alpha$ -mediated increase in BNP promoter activity. The inhibition of the ERK MAP kinase pathway with U0126, a MEK1/2 inhibitor, did not affect IL-1 $\beta$  and TNF- $\alpha$ -mediated increases in BNP promoter activity. On the other hand, U0126 completely abolished the ET-1 effect on BNP transcription. Collectively our results strongly suggest that pro-inflammatory

cytokines IL-1 $\beta$  and TNF- $\alpha$  directly increase BNP mRNA by transcription and that p38 MAP kinase is an important component in the activation of the transcription factor binding to DNA consensus enhancer elements on the BNP promoter.

To address whether the increase in mRNA abundance after IL-1 $\beta$  and TNF- $\alpha$  stimulation was due to post-transcriptional mechanisms we undertook experiments designed to disrupt any translation-dependent enhancement of mRNA stability. Unlike ANF mRNA, the 3'-untranslated region of BNP mRNA contains several AUUUA rich sequences (Kojima et al., 1989) that are known to modulate translation-dependent transcript stability for genes encoding cytokines and proto-oncogenes (Schiavi et al., 1992). This characteristic of the BNP gene is believed to be the reason behind the relatively short half-life of less than one hour for the BNP mRNA (Hanford et al., 1994) versus 30 hours for ANF mRNA (Gardner et al., 1988a). Stimulation with certain agonists such as phorbol esters (a protein kinase C agonist) (LaPointe and Sitkins, 1993), PE (Hanford et al., 1994) and ET-1 (Nakagawa et al., 1995) increases BNP mRNA stability. The increase in BNP mRNA stability caused by phorbol esters is sensitive to the protein synthesis inhibitor, cycloheximide suggesting the involvement of a protein that is responsible for rapid BNP mRNA turnover (LaPointe and Sitkins, 1993). The precise mechanism by which post-transcriptional processes increase BNP transcript abundance by other hypertrophic agonists is still currently unknown.

In our study, we report that post-transcriptional mechanisms are not playing a major role in the regulation of BNP mRNA turnover and abundance since cycloheximide did not alter IL-1 $\beta$  or TNF- $\alpha$ -induced increases in BNP mRNA.

Interestingly, others have shown that post-transcriptional mRNA stabilization is not a mechanism used by acute mechanical stretch to increase in BNP mRNA (Magga et al., 1997). Lastly, there is no evidence that IL-1 $\beta$  alters the mRNA half-life for IL-1 $\beta$ -stimulated expression of other genes such as skeletal  $\alpha$ -actin (Patten et al., 1996) or iNOS (LaPointe and Sitkins, 1996).

In summary, it is clear that pro-inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  can directly activate a rat -2.2-kbp BNP promoter. The precise secondary signaling pathway, transcription factors and DNA consensus elements that regulate this activity remain to be elucidated, but clearly from our studies the p38 MAP kinase pathway plays a predominant role. Some candidate *cis*-acting elements on the 5' BNP promoter include YY1 and M-CAT, but it is likely that there are others that have yet to be characterized. Lastly, the increased mRNA abundance caused by IL-1 $\beta$  and TNF- $\alpha$  is not due to increased transcript stability.

### **Both ANF and BNP secretion is increased by the hypertrophic agonist,**

#### **ET-1**

The discoordinate expression of ANF and BNP mRNA and secretion by pro-inflammatory cytokines is a novel observation since mechanical or neurohumoral agonists that cause an increase in ventricular release of the natriuretic peptides do so by increasing both ANF and BNP mRNA and secretion (Horio et al., 1993; Kinnunen et al., 1993). The insensitivity of ANF release to pro-inflammatory cytokines cannot be due to a possibility that our cell cultures did not have the ability to further upregulate ANF secretion because endothelin-1 (ET-1) at 10 nM stimulated the secretion of both natriuretic peptides from cell culture. ET-1 is a well-characterized

cardiocyte hypertrophic agonist and has been shown to increase expression of early response genes such as *c-fos* and *c-jun* proto-oncogenes as well as late activating genes such as ANF, BNP and  $\beta$ -MHC (Shubeita et al., 1990; Bruneau et al., 1997; Hilal-Dandan et al., 1997; Horio et al., 1998).

Immunoelectron microscopy of atrial cardiocytes have revealed that ANF and BNP are co-localized in mature secretory granules (Thibault et al., 1992; Mangat and de Bold, 1993). Therefore, it is not surprising that natriuretic peptide secretagogues cause the release of both ANF and BNP in isolated atrial preparations (Bruneau and de Bold, 1994) and in atrial cardiocyte cultures (Iida et al., 1990; Horio et al., 1993). However, it has also been demonstrated that ANF and BNP expression can be discoordinately regulated by  $\alpha$ -adrenergic agonists using isolated atrial preparations (Bruneau et al., 1996) and by acute versus chronic administration of DOCA-salt in a rat model of hypertension and cardiac overload (Yokota et al., 1995). An explanation for how two peptides that share a storage and secretory vesicle can at times be secreted discoordinately may be found when examining the secretory pathways used by cardiocytes to secrete the natriuretic peptides in the atria and ventricles. The predominant method of natriuretic peptide release in the atria is a regulated secretory pathway (Bloch et al., 1986) followed by a minor constitutive-like pathway (Ogawa et al., 1999). Regulated secretion is independent of protein synthesis and involves a concentrated packaging of secretory proteins into mature granules after exiting the *trans*-Golgi network (Burgess and Kelly, 1987). On the other hand, constitutive secretion is characterized by a rapid secretion of protein following synthesis without the requirement of granule formation that insensitive to stimulation. Constitutive-like secretion is described as exocytosis of secretory proteins from immature granules

independent of protein synthesis (Arvan et al., 1991; Kuliawat and Arvan, 1992). The discoordinate secretion of BNP in the atria may arise because BNP may utilize multiple secretory pathways since it has been demonstrated from tissue fractionation experiments that of newly synthesized proteins, only 8% of BNP as compared to 40% of ANF is associated with mature atrial granules (Bruneau et al., 1996). Therefore it is conceivable that a portion of BNP secretion by the atria can proceed through a granule-independent process that in specific conditions may lead to discoordinate secretion of ANF and BNP induced by agonists.

The precise secretory pathway by which ventricular cardiocytes increase natriuretic peptide release has not been clearly defined. Normal ventricular cardiocytes have fewer mature secretory granules as compared to atrial cardiocytes and thus it was initially proposed that a constitutive pathway may be primarily responsible for secretion of the natriuretic peptides by ventricular tissue (Bloch et al., 1986; De Young et al., 1994). However, the constitutive pathway may not be the sole pathway for the secretion of natriuretic peptides by the ventricles as there is evidence of a regulated secretory pathway, especially in disease states. In chronic cardiovascular overload in humans and using animal models of hypertrophy and congestive heart failure, it has been shown that the ventricle acquires an enhanced ability to store natriuretic peptides as evidenced by increased granule-associated natriuretic peptide immunoreactivity in these hearts (Ding et al., 1987; Edwards et al., 1988; Kinnunen et al., 1991). *In vitro*, NRVCs respond to hypertrophic stimuli like myocardial stretch (Liang and Gardner, 1998; Liang and Gardner, 1999; Liang et al., 2000b; Liang et al., 2001a), ET-1 (Shubeita et al., 1990; Irons et al., 1993) and PE (Hanford and Glembotski, 1996) by increasing natriuretic peptide secretion and

mRNA expression. Therefore, similar to the atria, the ventricles may also acquire the ability to selectively store and secrete BNP separate from ANF when BNP synthesis is increased by other agonists such as the pro-inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$ .

The absolute levels of ET-1-induced secretion ANF was greater than that for BNP (2523.2 $\pm$ 56.1 pg/ml and 772.5 $\pm$ 38.0 pg/ml, respectively) over 24 hours, but there was a larger fold increase of BNP than ANF (9.6-fold versus 4.7-fold increase over vehicle, respectively). The precise regulatory mechanism for the greater fold-increase in BNP secretion over ANF by certain hypertrophic agonists is not currently known, however, this phenomenon has also been reported by others using a similar cell culture model of ET-1 stimulated cardiocyte hypertrophy (Harada et al., 1999). Other examples where the fold-increase of BNP secretion has been greater than ANF have been documented by our laboratory using DOCA-salt animal models of hypertension and cardiac hypertrophy (Yokota et al., 1995) and others (Yokota et al., 1990). Humans with acute myocardial infraction (Mukoyama et al., 1991b) or severe congestive heart failure (Mukoyama et al., 1991a; Wei et al., 1993) there have also been instances where fold-increases of BNP secretion are greater than ANF. Based on the results of natriuretic peptide release by well-characterized hypertrophic agonists, it is reasonable to conclude that the regulation of BNP synthesis and secretion can at times be quite different from ANF.

In summary, ANF and BNP may be co-localized in the same mature secretory granule and in most instances both peptides are secreted coordinately by hypertrophic stimuli in the myocardium. In specific cases such as with ET-1 stimulation, BNP may

respond with a greater fold-activation than ANF but nonetheless ANF levels still remain greater than BNP, due to higher basal levels of ANF secretion. Although our studies were performed *in vitro* with NRVC cultures, it has been clearly established that these neonatal myocardial cells do respond similarly to their adult counterparts with respect to the increase in hypertrophic gene expression and thus may be seen as a clear reflection of the *in vivo* situation during pathophysiological conditions (Gardner et al., 1988b; Knowlton et al., 1991; Shubeita et al., 1992a; Argentin et al., 1994). Lastly, there may be differences in the storage and the secretory pathways between ANF and BNP in the atria and the ventricles. Therefore, cytokines like IL-1 $\beta$  and TNF- $\alpha$  that selectively upregulate BNP mRNA and protein synthesis, without affecting the expression of ANF, have in place a mechanism to permit the secretion of one hormone and not the other. The precise secretory pathway responsible remains to be elucidated.

### **IL-2, IL-6 and IFN- $\gamma$ do not alter natriuretic peptide secretion**

RT-PCR analysis has demonstrated the presence of pro-inflammatory IL-6 and immunomodulatory IL-2, and IFN- $\gamma$  cytokine expression during the acute phase of cardiac allograft rejection (Zhao et al., 1993; Cunningham et al., 1994). Therefore, we undertook experiments to examine the effects of these cytokines on the expression of natriuretic peptides from NRVC cultures. In our investigation, stimulation with IL-2, IL-6 and IFN- $\gamma$  at concentrations ranging from 0.5 to 100 ng/ml did not significantly affect either ANF or BNP secretion after 48 hours.

Little information is known about the effects of IL-6, IL-2 and IFN- $\gamma$  on cardiac gene expression and function, but of this group, IL-6 and the IL-6-type

cytokines are the best studied. IL-6 is a member of the IL-6-type family of cytokines that all share a common gp130 subunit. The gp130 glycoprotein mediates signaling predominately via activation of the JAK-STAT pathway and partially by the MAP kinase pathways (Hirano et al., 2000). IL-6 stimulation is negatively inotropic and may decrease intracellular  $Ca^{2+}$  concentrations via stimulation of nitric oxide synthesis and cGMP pathways in ventricular cardiocytes (Finkel et al., 1992). IL-6 is not expressed in the normal heart, but IL-6 mRNA can be induced by other cytokines such as TNF- $\alpha$  (Gwechenberger et al., 1999) and this secreted IL-6 has been reported to act in an autocrine manner to protect against sphingosine-induced cardiocyte apoptosis (Craig et al., 2000). However, there is evidence that IL-6 alone may not be capable of mediating changes in cardiac gene expression since mice with overexpression of IL-6 protein develop hypertrophy only in the presence of constitutively active IL-6 receptors (Hirota et al., 1995). Other members of the IL-6-type cytokines such as cardiotrophin-1 (CT-1) and leukemia inhibitory factor (LIF) robustly promote sarcomeric organization and increased natriuretic peptide gene expression. CT-1 and/or LIF increase protein synthesis (Kodama et al., 1997) BNP (Kuwahara et al., 1998; Hamanaka et al., 2000) and ANF mRNA via the JAK-STAT pathway (Wollert and Chien, 1997; Kunisada et al., 1998). Overall, these reports from other laboratories generally conclude that the IL-6-type cytokines can activate genes that are hallmarks of hypertrophy, including sarcomeric reorganization and ANF and BNP gene expression. In summary, although data from our experiments show that IL-6 did not increase natriuretic peptide secretion after 48 hours, it is possible that IL-6 is not capable of working alone and collaboration with other cytokines is required to mediate changes in cardiac natriuretic peptide gene expression.

The effects of IL-2 and IFN- $\gamma$  on cardiac gene expression and function are largely uncharacterized. It is known that both signal to the nucleus predominately using the JAK-STAT pathway in cells of the immune system (Darnell, Jr., 1998), but may also use the SAPK kinases and phosphatidylinositol 3-kinase (PI3K) and numerous secondary signaling pathways (Platanias and Fish, 1999; Uddin et al., 1999; Ellery and Nicholls, 2002).

### **Cytokine combinations can increase BNP secretion**

As mentioned in the previous section, IL-6, IL-2 or IFN- $\gamma$  were not able to stimulate the secretion of natriuretic peptides from NRVC cultures. However, since the biological effects of immune activation are often the result of sequential production and action of several cytokines on target cells, we also tested a combination of cytokines for their effect on ANF or BNP secretion. This idea is based on the hypothesis that the priming of signaling machinery within the cardiocyte by multiple cytokines may be necessary in the case of cytokines such as IL-6, IL-2 or IFN- $\gamma$  to stimulate a change in BNP expression. In support of this idea, there is strong evidence that for IFN-mediated signaling to elicit maximal activation of transcription by the STAT transcription factors, STATs require additional serine phosphorylation by the MAP kinases (David et al., 1995; Wen et al., 1995). In the pancreatic islets of Langerhans, it has been shown that IFN- $\gamma$  potentiates IL-1 $\beta$ -induced ERK MAP kinase activity (Andersen et al., 2000). IL-1 $\beta$  can also increase STAT phosphorylation, but it appears that this phosphorylation is most likely a secondary effect due to the induction of cytokines or their receptors that utilize the JAK-STAT pathway (Ng et al., 2001). Therefore, there is evidence to suggest that cytokines like

IFN- $\gamma$  may cooperate directly and indirectly with other cytokines to modulate natriuretic peptide gene expression.

To examine whether IFN- $\gamma$  required additional activation by MAP kinases we co-stimulated NRVC cultures with IFN- $\gamma$  and IL-1 $\beta$ , a potent stimulator of p38 MAP kinase. Our results show that although IFN- $\gamma$  alone does not significantly increase BNP secretion, IFN- $\gamma$  when used in combination with IL-1 $\beta$  results in a synergistic increase in BNP secretion that far exceeds the levels of secretion induced by only IL-1 $\beta$ .

In summary, the implications from this experiment are important because it demonstrates that IL-1 $\beta$  signaling can cooperate with IFN- $\gamma$ -mediated induction of BNP expression and that there is cross-talk from multiple cytokine initiated secondary pathways to synergistically increase the expression of BNP.

### **Mixed lymphocyte reaction conditioned medium increases BNP secretion**

In this study, we used a novel approach designed to circumvent the omission of any pro-inflammatory or immunomodulatory cytokines or their combinations associated with cardiac transplant rejection that may have BNP stimulating properties. NRVC cultures were stimulated with conditioned medium from an allogenic mixed lymphocyte reaction (MLR). The unidirectional allogenic MLR was suggested to be an independent assay for the prediction of transplant rejection (Harmon et al., 1982; Langhoff et al., 1985). At present, it is now believed that the MLR may only have limited prognostic potential in transplantation (Steinmann et al., 1994) and only be of

benefit if used with human leukocyte antigen (HLA) typing (Cartwright et al., 2000). Nonetheless, the MLR remains the only *in vitro* model of transplantation immunity reflecting complex interactions of lymphocytes and is still used extensively for studying profiles of cytokine production and secretion during acute or chronic inflammation (Zeevi et al., 1980; Jordan and Ritter, 2002).

For the MLR, lymphocytes were harvested from two different inbred rat strains from secondary lymphoid tissues such as cervical or axillary lymph nodes where mature lymphocytes are found in high concentrations. One cell population was designated the responder lymphocytes and was incubated with the stimulator lymphocytes, which were rendered incapable of proliferation by  $\gamma$ -irradiation. In general, allorecognition begins with presentation of foreign antigens by the MHC class II receptors on passenger leukocytes (or dendritic cells). The MHC class II receptor is recognized by T-cell receptor (TCR) on the  $T_H$ -lymphocyte leading to lymphocyte activation. These responding cells then undergo morphologic and biochemical changes that include cell proliferation and secretion of numerous lymphokines (Dupont and Hansen, 1976). Based on the current information, cytokines that are released in the MLR are IL-1 $\beta$ , TNF- $\alpha$ , IFN- $\gamma$ , IL-2, IL-4, IL-6, IL-10, IL-12, IL-18 and LIF (Schmitt and Schenkein, 1983; Pure et al., 1988; Leenaerts et al., 1992; Baan et al., 1994; Causey et al., 1994; Daane et al., 1994; van Emmerik et al., 1994; Bishara et al., 1998; Kohka et al., 1999; Jordan and Ritter, 2002). However, there is no absolute agreement on the precise cytokines or the total number that may be secreted in an MLR due potentially to a variety of reasons. Many of the aforementioned studies have used human or rat lymphocytes and there is potential for species differences in the MLR response. Other differences may arise due to the

source of lymphocytes, since in some of the animal studies and all the human studies, lymphocytes were harvested from peripheral blood mononuclear cells (PBMC) and not from secondary lymphoid tissues. Lastly, there have been instances where mitogenic stimulation with mitomycin C was used instead of generating an allo-reactive biological response with stimulator and responder cell populations, which has produced conflicting results even from within the same laboratory (Cartwright et al., 2000).

In this study, we report that at the end of a 5-day MLR culture there was a significant increase in  $^3\text{[H]}$ -thymidine incorporation from the MLR group with respect to the responder or stimulator control groups confirming T-lymphocyte activation and proliferation caused by an allogenic reaction. Importantly, serum-free MLR conditioned medium (SF-MLR-CM) from this 5-day reaction when applied to NRVC cultures in increasing proportions relative to the basal DMEM cardiocyte culture medium resulted in a 4 to 4.5-fold increase in BNP secretion over the control groups after 48 hours of stimulation. It is interesting to note that the fold-increase in BNP secretion by the SF-MLR-CM was significantly greater than either IL-1 $\beta$  or TNF- $\alpha$  alone (4 to 4.5-fold BNP increase with SF-MLR-CM versus 2 to 2.5-fold BNP increase with IL-1 $\beta$  or TNF- $\alpha$ ). In contrast, not even the highest concentration of SF-MLR-CM altered ANF secretion. The BNP production is specific to the allo-activated MLR, as conditioned medium from non-activated lymphocytes did not significantly increase BNP or ANF secretion. Lastly, we examined the signaling mechanism responsible for mediating the increase in BNP gene expression after stimulation with SF-MLR-CM. Our results suggest that the increase in BNP by the SF-MLR-CM is dependent on signaling from p38 MAP kinase since SB203580

completely suppressed any MLR-induced increase in BNP secretion. Basal ANF and BNP secretion was unaffected by SB203580 treatment.

In summary, in this section we show that conditioned medium from a 5-day MLR significantly stimulated BNP secretion from neonatal rat ventricular cardiocyte cultures. The increase was specific for BNP, as ANF secretion was not altered even in the presence of the high concentrations of SF-MLR-CM. With this novel approach we attempted to replicate the conditions seen during transplant rejection using an established *in vitro* model of transplantation immunity. Lastly, we also showed that the secondary-signaling pathway used by the SF-MLR-CM to selectively stimulate BNP secretion is the p38 MAP kinase.

### **IL-1 $\beta$ and TNF- $\alpha$ are not mediating MLR-induced increases in BNP secretion**

As mentioned in the previous sections, a large number of pro-inflammatory or immunomodulatory cytokines are present during inflammation associated with an allograft rejection episode. Many of these cytokines can also be found produced *in vitro* by the MLR. In this study, the SF-MLR-CM stimulated a larger increase in BNP secretion than the individual cytokines IL-1 $\beta$  and TNF- $\alpha$  and thus raised the possibility that these cytokines may be partly involved in MLR-induced BNP gene expression.

To determine if IL-1 $\beta$  and TNF- $\alpha$  were present in the SF-MLR-CM, the conditioned medium was collected after a 5-day reaction and assayed using a sandwich ELISA technique. Despite repeated attempts, we were unable to detect rat

IL-1 $\beta$  or TNF- $\alpha$  in the SF-MLR-CM within the detection limit of the ELISA (~5 pg/ml). As previously mentioned, a few reports have detected IL-1 $\beta$  and TNF- $\alpha$  in appreciable amounts from human MLR conditioned medium (Jordan and Ritter, 2002), but other studies do not report that IL-1 $\beta$  and TNF- $\alpha$  constitute any significant percentage of cytokine in SF-MLR conditioned medium (Pure et al., 1988). Nevertheless, despite the debate as to the concentration of IL-1 $\beta$  or TNF- $\alpha$  that may be present in the MLR-CM, our previous experiences indicate that even small amounts of a given cytokine in combination with other cytokines may elicit a maximal response in BNP gene expression. Therefore, an additional experiment was designed to eliminate any effects caused by trace levels of IL-1 $\beta$  or TNF- $\alpha$  that may be acting in concert with a combination of other cytokines that cannot readily be detected using the ELISA technique.

It has been shown that the effects of IL-1 $\beta$  and TNF- $\alpha$  signaling in cardiocytes and other cells can be suppressed by the addition of soluble receptor antagonists to IL-1 $\beta$  and TNF- $\alpha$  receptors (Ungureanu-Longrois et al., 1995; Inoue et al., 1999; Mariani et al., 1999). In our experiments, interleukin-1 receptor antagonist (IL-1RA) and the soluble TNF receptor I and II (sTNF-RI/RII) were used to suppress the actions of IL-1 $\beta$  and TNF- $\alpha$ , respectively. IL-1RA is a 25-kDa protein that binds with high affinity (200 pM) to the active type-I interleukin-1 receptor (IL-1RI) in a competitive manner to IL-1 $\beta$  which it shares 26% homology (Eisenberg et al., 1990). The recombinant soluble TNF receptors I/II are 21 and 25-kDa proteins that compete with the endogenous TNF receptors, TNF-RI and TNF-RII respectively (Grell, 1995; Aderka, 1996). Our results show that the addition of 1  $\mu$ g/ml of each IL-1RA and sTNF-RI/RII did not suppress the MLR conditioned medium-induced increases in

BNP secretion. This suggests that IL-1 $\beta$  and TNF- $\alpha$  may not be the primary effectors in our MLR and there are factor(s) yet to be characterized in the conditioned medium that have the ability to selectively upregulate BNP production.

In summary, to our knowledge, this study is the first to report the finding that lymphokines or other stimulating factors present in conditioned medium from allo-activated mixed lymphocyte reaction cultures can selectively modify BNP gene expression in ventricular cardiocytes. We attempted to determine if IL-1 $\beta$  and TNF- $\alpha$  are major constituents in our SF-MLR-CM, but the results of the ELISA as well as experiments with the soluble IL-1RA and sTNF-RI/II strongly suggest that IL-1 $\beta$  and TNF- $\alpha$  are not present in our SF-MLR-CM. It remains for future studies to determine the precise component(s) of our MLR conditioned medium that are responsible for the selective upregulation of BNP. Importantly, however, this model offers a new paradigm for which to study natriuretic peptide gene expression in inflammatory conditions.

## CONCLUSIONS

In this study, we showed that a discoordinated expression and secretion of the natriuretic peptides ANF and BNP is seen after exposure to pro-inflammatory cytokines IL-1 $\beta$  or TNF- $\alpha$ . Stimulation of neonatal ventricular cardiocytes with IL-1 $\beta$  and TNF- $\alpha$  upregulated BNP mRNA and secretion while ANF mRNA and secretion remain unchanged with respect to basal conditions. The failure of IL-1 $\beta$  and TNF- $\alpha$  to increase ANF gene expression was not due to an inability of cultured ventricular cardiocytes to secrete ANF, since stimulation of the cultures with the hypertrophic agonist ET-1, expectedly increased both ANF and BNP secretion.

The upregulation of BNP mRNA is transcription-dependent because actinomycin D completely suppressed the BNP mRNA increase induced by IL-1 $\beta$  and TNF- $\alpha$ . The 3'-untranslated region of the BNP mRNA is very different from the other natriuretic peptides because it contains numerous AUUUA rich sequences that may confer translation-dependent mRNA stability. However, we determined that post-transcriptional BNP mRNA stabilization mechanisms are not likely to be responsible for the increased mRNA abundance since cycloheximide did not suppress the stimulatory effects of IL-1 $\beta$  and TNF- $\alpha$  on BNP mRNA.

The signal transduction pathway used by IL-1 $\beta$  and TNF- $\alpha$  to increase BNP gene expression is the stress-activated p38 MAP kinase. Within 15 minutes of stimulation, there was a rise in phosphorylated p38 MAP kinase abundance and activity. Transient transfection of -2.2-kbp BNP promoter revealed that IL-1 $\beta$  and

TNF- $\alpha$  directly stimulate promoter activity. This activity is highly dependent on signaling by p38 MAP kinase, since pre-treatment of cardiocytes with a specific p38 inhibitor, SB203580 abolished IL-1 $\beta$  and TNF- $\alpha$ -induced increase in BNP promoter activity, mRNA and protein secretion. Basal ANF and BNP secretion are not dependent on p38 MAP kinase signaling.

Other soluble factors present in abundance during inflammation and allograft rejection are IL-6, IL-2 and IFN- $\gamma$ . Individual stimulation of NRVCs with these cytokines (up to the maximal 100 ng/ml) did not alter natriuretic peptide expression. However, when a combination of both IFN- $\gamma$  and IL-1 $\beta$  is introduced, it was demonstrated that the increase in BNP secretion was greater than that of IL-1 $\beta$  alone. This strongly suggests that mechanisms exist for pro-inflammatory cytokines to cooperatively promote the induction of BNP.

Lastly, we show that serum-free MLR conditioned medium obtained from allo-activated lymphocyte cultures was able to increase BNP secretion from cultured ventricular cardiocytes and this effect can be prevented by using an inhibitor to p38 MAP kinase.

### **Physiological Implications for Increased Plasma Levels of BNP During Heart Transplant Rejection**

Overall, from the above results, it may be reasonably concluded that the selective increase in plasma BNP concentration observed during an overt inflammatory response seen in the acute phase of cardiac allograft rejection can be at least partly explained by the ability of pro-inflammatory cytokines to selectively

modulate cardiocyte gene expression. However, the biological relevance, for the increased baseline levels of BNP and ANF plasma levels after heart transplantation and the even greater levels of BNP during rejection is not immediately apparent. It is interesting to speculate that in the working heart, the increase in BNP (and ANF) in plasma may be beneficial and may be acting in an autocrine and/or paracrine manner on fibroblast, endothelial and immune cells to maintain and improve cardiac performance of the allograft.

Over time the transplanted heart may develop mild forms of cardiac hypertrophy but the mechanisms responsible for this are currently ill defined (Rowan and Billingham, 1992; Armstrong et al., 1998). It has been reported that the persistent local expression of pro-inflammatory cytokines such as TNF- $\alpha$  is associated with post-heart transplantation cardiac hypertrophy (Stetson et al., 2001). Furthermore, it is also known that cardiac overexpression of TNF- $\alpha$  in mice leads to cardiac hypertrophy and subsequent heart failure (Bryant et al., 1998). Therefore, in addition to a major role in maintaining salt and water balance, the natriuretic peptides may limit myocardial cell growth, a property that may be especially important after cardiac transplantation. ANF and BNP limit endothelial (Itoh et al., 1992), vascular smooth muscle cell growth (Itoh et al., 1990; Hutchinson et al., 1997) and inhibit vasoactive agonist-induced cardiocyte hypertrophy (Silberbach et al., 1999; Rosenkranz et al., 2003). It is important to keep in mind that a direct correlation between pro-inflammatory cytokines and hypertrophy in the post-transplanted heart has yet to be proven with a larger study than that of Stetson *et al.* (Stetson et al., 2001). Nonetheless, it is conceivable that increased local BNP secretion due to increased

immunological activity, might be acting to limit the amount of cardiocyte hypertrophy and to preserve cardiac function.

Diastolic dysfunction and decreased cardiac compliance due to the development of cardiac fibrosis has been reported after heart transplantation in humans (Pickering and Boughner, 1990; Rowan and Billingham, 1990; Tazelaar et al., 1990) and in animal models (Kolar et al., 1996). The mechanisms responsible are not clear, but there is evidence to support the idea that pro-inflammatory cytokines may directly or indirectly stimulate cardiac fibrosis. In the intact heart, TNF- $\alpha$  has been shown to induce expression of ANG-II, a well-known mediator of fibrosis (Kapadia et al., 1998). Furthermore, mice with cardiac restricted overexpression of TNF- $\alpha$  rapidly develop cardiac fibrosis and dilated cardiomyopathy (Bryant et al., 1998). The first indication that natriuretic peptides, specifically BNP, may possess properties to counteract cardiac fibrosis was suggested from mouse BNP knockout experiments. BNP<sup>-/-</sup> mice develop focal fibrotic lesions at 20 weeks of age in the absence of systemic hypertension and cardiac hypertrophy (Tamura et al., 2000). To investigate this idea further, Ogawa *et al.* demonstrated that the antifibrotic effects of BNP arise from its ability to specifically inhibit cardiac fibroblast proliferation (Ogawa et al., 2001). Indeed, more recent studies have shown that BNP signaling directly inhibits FGF-induced increases in collagen I, fibronectin proteins, tissue inhibitor of matrix metalloproteinases (TIMP) and other proteins involved in the cardiocyte growth such as insulin growth factor 1 (IGF1) and platelet derived growth factor A (PDGFA) (Kapoun et al., 2004). Therefore, the increased BNP release from cardiocytes as a result of acute or chronic exposure to pro-inflammatory cytokines may act as a limiting factor in the development of fibrosis in the transplanted heart.

The precise signaling pathways used by BNP to inhibit cardiac fibrosis are not known. ANF and BNP binding to the NPR-A receptor predominantly leads to the formation of cGMP and activation of cGMP-dependent protein serine/threonine kinases. It has been suggested that the antihypertrophic activity of the natriuretic peptides result from cGMP-dependent kinase inhibition of ERK MAP kinase pathway (Rosenkranz et al., 2003). However, ANF has not been shown to have antifibrotic properties. Thus, it remains unclear how BNP may exclusively possess these properties when both BNP and ANF share a common biological receptor, NPR-A. Recent NPR-A knockout mouse experiments have reported that BNP activity remains in some specific cell types even in the absence of detectable ANF activity (Goy et al., 2001). These authors allude to the existence of a receptor that may preferentially bind BNP over ANF. Furthermore, it has been speculated that BNP can at times signal through receptor pathways that are not invoked by ANF, although which specific pathway is not known (Chusho et al., 2000). It is important to bear in mind that these reports are preliminary, and additional experiments are required to better understand the role played by increased BNP levels in the transplanted and rejecting heart and the biochemical mechanisms underlying this unique property of BNP.

The natriuretic peptides have also been shown to inhibit components of the immune system. ANF can inhibit lipopolysaccharide (LPS) (Kierner et al., 2000) or IFN- $\gamma$ -induced (Tsukagoshi et al., 2001) macrophage derived IL-1 $\beta$  and TNF- $\alpha$  expression and secretion by interfering with p38, NF- $\kappa$ B and activation protein-1 (AP-1) activation in a cGMP-dependent manner (Kierner and Vollmar, 2001; Kierner et al., 2002). It has also been shown that ANF can suppress the expression of

inducible nitric oxide synthase (iNOS) mRNA in macrophages which is particularly important since NO can decrease cardiac contractility (Finkel et al., 1992).

Lastly, the present work examined the role of pro-inflammatory cytokines that are present during heart transplantation and allograft rejection on the expression of BNP and ANF from an isolated and purified cardiocyte cell culture system. It is important to keep in mind that a working heart is not a homogeneous population of cardiocytes. Cardiocytes comprise 70-80% of heart mass, but only 20-30% of total cardiac cells (Hefti et al., 1997). As such, interactions between cardiocytes and non-cardiocytes exist. Another layer of complexity arises during heart transplantation and rejection where there are interactions between myocardial and immune cells. The mixed lymphocyte reaction is an established *in vitro* representation of transplantation immunity. The application of conditioned medium from allo-activated T-lymphocyte cultures to cardiocyte cell cultures is a good model to study the mechanisms by which pro-inflammatory cytokines regulate the expression of the natriuretic peptides and in general, cardiac gene expression and function.

In summary, the precise role that increased natriuretic peptides levels are playing in the transplanted heart is unknown. Moreover, it remains to be determined if increased levels of BNP preceding and during an allograft rejection episode are acting to maintain and preserve cardiac function during stress. A complete understanding of the mechanisms by which pro-inflammatory cytokines and transplant rejection regulate the expression of BNP may provide a basis for exploring the possibility that BNP may be used as a non-invasive tool to diagnose allograft rejection.

## Future Directions

The precise mechanisms by which pro-inflammatory cytokines specifically activate the BNP but not the ANF promoter has not been formally addressed in this study. From the preceding sections, it is reasonable to suggest that there may be *cis*-acting elements present on the BNP promoter but not on the ANF promoter, that are responsive to stimulation by pro-inflammatory cytokines IL-1 $\beta$ , TNF- $\alpha$  and conditioned medium from MLRs. Furthermore, the exact component(s) in the MLR conditioned medium that are responsible for selectively upregulating BNP gene expression over ANF gene expression have not been characterized.

In order to accomplish the first goal, 5'-sequential truncation mutants of the BNP promoter generated by PCR will be required. Mutant(s) demonstrating a significant decrease in BNP activity after stimulation would be screened using electronic software such as MatInspector™ to identify the *cis*-element(s). Single point mutations of the *cis*-acting element could be performed using the Quikchange® system. Additionally, *in vitro* electrophoretic mobility shift assays (EMSA) using oligonucleotide probes resembling this *cis*-acting element will be performed with supershift assays to characterise the *trans*-acting protein.

To accomplish the second goal of determining the identity of the cytokine within the MLR conditioned medium possessing BNP-inducing properties would be to subject the MLR conditioned medium to immunoaffinity adsorption to remove the candidate pro-inflammatory cytokine. Alternatively, a HPLC-based approach could also be used to separate the components of the MLR conditioned medium.

In conclusion, our investigation introduces a novel area of study concerning cardiac NPs during inflammation and the mechanism of selective regulation of BNP gene expression. Our work makes novel contributions, since the common belief developed from experiments with simulation by conventional hypertrophic agonists was the simultaneous increase in both ANF and BNP gene expression and secretion. Our results show that certain cytokines can directly upregulate BNP transcription and translation without affecting ANF. These results allude to important differences in the regulation of secretion and gene expression of these two cardiac-derived natriuretic hormones by pro-inflammatory cytokines.

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