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B-Type Natriuretic Peptide and Extracellular Matrix Protein Interactions in Human Cardiac Fibroblasts

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Abstract

Cardiac fibroblasts (CFs) regulate myocardial remodeling by proliferating, differentiating, and secreting extracellular matrix (ECM) proteins. B-type natriuretic peptide (BNP) is anti-fibrotic, inhibits collagen production, augments matrix metalloproteinases, and suppresses CF proliferation. Recently, we demonstrated that the ECM protein fibronectin (FN) augmented production of BNP's second messenger, 3', 5' cyclic guanosine monophosphate (cGMP) in CFs, supporting crosstalk between FN, BNP, and its receptor, natriuretic peptide receptor A (NPR-A). Here, we address the specificity of FN to augment cGMP generation by investigating other matrix proteins, including collagen IV which contains RGD motifs and collagen I and poly-L-lysine, which have no RGD domain. Collagen IV showed increased cGMP generation to BNP similar to FN. Collagen I and poly-L-lysine had no effect. As FN also interacts with integrins, we then examined the effect of integrin receptor antibody blockade on BNP-mediated cGMP production. On FN plates, antibodies blocking RGD-binding domains of several integrin subtypes had little effect, while a non-RGD domain interfering integrin α 5 β 1 antibody augmented cGMP production. Further, on uncoated plates, integrin α 5 β 1 blockade continued to potentiate the BNP/cGMP response. These studies suggest that both RGD containing ECM proteins and integrins may interact with BNP/NPR-A to modulate cGMP generation.

Formation of the extracellular matrix (ECM) is essential for growth and favorable fibrosis, however, dysregulation of this process leads to deleterious consequences. Cardiac fibrosis is a hallmark of myocardial dysfunction produced by mechanical and/or hormonal stimulation (Weber and Brilla, 2001; Jugdutt, 2003), leading to heart failure (HF). The long-term consequences of cardiac fibrosis secondary to ECM remodeling include impaired myocardial function, tissue hypoxia and arrhythmogenesis (Frank and Langer, 1974; Burlew and Weber, 2002; Kohl, 2003). While cardiomyocytes (CMCs) occupy the greatest volume within the heart, cardiac fibroblasts (CFs) are the most abundant cell type and contribute to myocardial remodeling and fibrosis by proliferating, differentiating, and secreting ECM proteins (Porter and Turner, 2009). Thus, the CF has become a therapeutic target in the treatment and prevention of numerous cardiovascular diseases including HF (Zannad et al., 2001; Pitt, 2002; Pitt et al., 2003).

The cardiac secretory peptide B-type natriuretic peptide (BNP) is a ligand for the natriuretic peptide receptor A (NPR-A), which when activated, results in the generation of the second messenger 3', 5'-cyclic guanosine monophosphate (cGMP) (Garbers et al., 2006). While thought originally to serve only as a circulating factor participating in cardiorenal regulation,

more recent investigations establish a paracrine role for BNP in the heart to regulate CMC growth and function as well as contribute to angiogenesis (Silberback and Roberts, 2001; Holtwick et al., 2003; Patel et al., 2005; Kuhn's JCI paper from 2009). Additionally, disruption of the BNP gene in the mouse results in load independent cardiac fibrosis and genetic deletion of NPR-A results in a model of exaggerated fibrosis in response to an increase in load (Tamura et al., 2000; Patel et al., 2005).

More recently, the fibro-inhibiting properties of BNP have been demonstrated. Others and we have reported that BNP is synthesized in CFs and inhibits collagen synthesis and CF proliferation (Tsuruda et al., 2002; Horio et al., 2003; Huntley et al., 2006). In addition, evidence has also suggested that BNP inhibits TGF-beta activation of pro-fibrotic and inflammatory genes in cultured human CFs (Kapoun et al., 2004). Furthermore, it has been reported that NPR-A receptors might transduce signals from the ECM in CFs (Liang et al., 2000; Huntley et al., 2006). Specifically, we observed that CFs plated on fibronectin (FN) demonstrated a pronounced increase in BNP-induced cGMP production compared to non-coated plates (Huntley et al., 2006). This action was further enhanced by a novel Mayo designed and synthesized NPR-A-specific RGD peptide. We speculated this enhanced cGMP production may involve binding of the RGD domain in FN to the NPR-A receptor, similar to RGD-binding integrin receptors. Such crosstalk in CFs between FN and NPR-A in BNP-induced activation of cGMP complements reports by Liang et al. (2000), which suggested outside-inside signaling in CMCs. Specifically, these investigators reported that CMCs grown on FN-coated plates increased BNP gene expression with increased amplification in response to CMC stretch. Importantly, a synthetic integrin blocking RGD sequence containing peptide competing for cell matrix interactions resulted in a dose-dependent reduction in BNP promoter activity. These studies suggested that the ECM environment of a cell may impact its response to pathological stimulations via either integrin or NPR-A receptors.

Integrins, like NPR-A, are dimeric receptors composed of alpha and beta subunits which bind to ECM proteins such as FN, collagen, and fibrillin (Johansson et al., 1997; Ross, 2002; Wiesner et al., 2005; Humphries et al., 2006). These ECM proteins influence integrin activity, transducing information from the external environment of the cell inside, where it influences cell activity (Hsueh et al., 1998). Integrin activation can also regulate components of the ECM, inducing the expression of metalloproteinases and other enzymes that regulate the amount and type of proteins in the ECM (Hsueh et al., 1998; Esparza et al., 1999). Through these interactions, RGD-dependent integrins have been shown to play a role in CF actions that contribute to remodeling in the heart. In particular, integrin $\alpha_v\beta_3$ is expressed by CFs and participates in CF proliferation and migration which contribute to cardiac remodeling (Graf et al., 2000; Ross and Borg, 2001).

In the current study, we sought to determine if the enhancement in cGMP generation is specific to FN or whether other ECM proteins such as collagen I and collagen IV, or the synthetic protein poly-L-lysine also modulate cGMP generation in CFs. Specifically, we hypothesized that collagen IV, an important component of basement membranes that binds to RGD-binding integrin receptors, but not collagen I or poly-L-lysine which do not contain RGD domains, will augment BNP activation of cGMP. We also explored potential crosstalk between NPR-A and integrin receptors through RGD-DDX interaction in BNP-induced cGMP generation by performing studies in the presence and absence of antibodies to integrin receptor subtypes to further delineate the role of these receptors in BNP generation of cGMP.

Methods

Cell culture

Human CFs (ScienCell, San Diego, CA) were cultured in manufacturer fibroblast media (ScienCell) supplemented with fibroblast growth serum (FGS), fetal bovine serum (FBS), and penicillin/streptomycin supplied as supplements with the media on uncoated, FN, collagen I, collagen IV, or poly-L-lysine coated plates (Becton Dickinson, Bedford, MA). Cells were treated at 80–90% confluency. Only cell passages 2 through 4 were used for experiments (Tsuruda et al., 2002; Huntley et al., 2006).

Cyclic GMP assay

Cells were assayed as described previously (Huntley et al., 2006). Briefly, cells were incubated serum free in Hank's balanced salt solution (Invitrogen, Carlsbad, CA) containing 20 mmol/L N-[2-hydroxyethyl]piperazine-N'[2-ethanesulfonic acid], 0.1% bovine serum albumin, and 0.5 mmol/L 3-isobutyl-1-methylxanthine (Sigma, St. Louis, MO). Treated cells received 10^{-6} M human BNP (Phoenix Pharmaceuticals, Inc., Burlingame, CA) for 10 min with or without various RGD-binding integrin-specific antibodies; anti-plasma fibronectin cell binding (FNCB) peptide antibody; anti-integrin α v β 3 antibody; anti-integrin β 1 adhesion blocking antibody; or anti-integrin β 3 antibody (Chemicon, Temecula, CA). Cells were lysed in 6% TCA and sonicated for 10 min. The samples were ether extracted four times in four volumes of ether, dried, and reconstituted in 300 μ l cGMP assay buffer. The samples were assayed using a competitive RIA cGMP kit (Perkin-Elmer, Boston, MA). Briefly, samples and standards are incubated with 100 μ l anti-human cGMP polyclonal antibody and 125 I-antigen for 18 h. Cyclic GMP assay buffer was added to the samples and they were centrifuged for 20 min at 2,500 rpm. The free fraction was aspirated off and the bound fraction was counted and concentrations determined. Samples are corrected for dilution factors and protein concentration, and values are expressed as pmoles/ml. There is no cross-reactivity with ANP, BNP, CNP, ET, and <0.001% cross-reactivity with cAMP, GMP, GDP, ATP, GTP.

All data are expressed as mean $\pm$ SEM. Unpaired Student's t-test was used for comparison between groups. Statistical significance was accepted at $P < 0.05$.

Results

Cyclic GMP generation in human cardiac fibroblasts: Modulation by ECM proteins

Based on our previous findings that FN augmented cGMP production by BNP, we evaluated the ability of three additional matrix proteins to affect cGMP production by BNP. As shown in Figure 1, CFs plated on FN, collagen I, collagen IV, or poly-L-lysine-coated plates, in the absence of BNP, did not generate cGMP. In contrast, BNP alone on uncoated plates significantly augmented cGMP generation. This action was significantly potentiated when CFs were plated on FN. Collagen IV-coated plates also increased the response to BNP. In contrast, collagen I and poly-L-lysine had no modulating action on cGMP production in cultured human CFs.

Cyclic GMP generation in human cardiac fibroblasts: Affect of integrin receptor subtype antibody blockade on FN-coated plates

Figure 2 illustrates the affect of various integrin-specific subtype antibodies (1:100 dilutions) on BNP-induced cGMP production on FN-coated plates. Integrin receptor inhibition using a FNCB integrin antibody that inhibits binding to FN did not affect BNP stimulated cGMP production. Blockade with either integrin β 1 antibody or integrin β 3 antibody, both of which block binding at RGD on FN, did not modulate BNP-induced

cGMP production. In contrast, integrin receptor blockade with an antibody to integrin $\alpha_v\beta_3$ that blocks RGD ligands, while not directly interacting with the RGD site significantly enhanced cGMP production.

Cyclic GMP generation in human cardiac fibroblasts: Affect of integrin receptor subtype antibody blockade on uncoated plates

To separate the effects of integrin antibody blockade from those of the FN coating, we examined the affect of integrin antibody blockade on CFs plated on uncoated plates. As shown in Figure 3, integrin $\alpha_v\beta_3$ antibody blockade dose dependently significantly enhanced BNP-induced cGMP production. The enhanced production by integrin $\alpha_v\beta_3$ antibody blockade was greater with FN coating than without, suggesting an additive affect (Fig. 3). In addition, antibody blockade with integrin $\alpha_v\beta_3$ combined with NPR-A RGD peptide was also synergistic (Fig. 4).

DISCUSSION

The goal of the current study was to provide new insights into the relationship between BNP, NPR-A, ECM proteins, integrins, and cGMP production in human CFs.

First, we reconfirmed our previous observation in human CFs that FN significantly enhances BNP-induced cGMP production. To broaden our previous findings and address the specificity of collagen and BNP interaction, we examined three additional proteins, the major collagen, collagen I which is the most abundant collagen in the heart (Taubenberger et al., 2010); collagen IV, which can bind to RGD-binding integrin receptors and is abundant in the normal heart (Ogawa et al., 2000; Pedchenko et al., 2004); and poly-L-lysine, which does not interact with cell surface receptors (McKeehan, 1984). Collagen I showed no statistical affect on BNP stimulated cGMP generation, collagen IV increased BNP stimulated cGMP similar to FN, while poly-L-lysine had no effect on BNP stimulated cGMP generation. Next, we defined the involvement of FN and RGD-binding integrin receptors in this enhanced BNP-induced cGMP effect. We demonstrate that blockade of several integrins, including FNCB, integrin β_1 and integrin β_3 , had no effect on BNP-induced cGMP production in CFs, while integrin $\alpha_v\beta_3$ receptor blockade enhanced cGMP production both in the presence and absence of FN. These studies advance the concept that ECM proteins which possess RGD motives can influence NPR-A activation by BNP in CFs, and that BNP-induced cGMP can be modified by an integrin receptor, strongly supporting the concept of outside-inside signaling by the BNP/NPR-A/cGMP pathway.

In our previous report, we hypothesized that FN, either through direct NPR-A binding, possibly via RGD motifs of FN interacting with NPR-A receptors, or through integrin receptor participation by an undetermined mechanism, enhances cGMP generation when BNP activates the NPR-A. Here, we demonstrate that collagen I, which does not interact with RGD motifs in its native state, did not affect BNP stimulated cGMP production. Of interest, thermal or proteolytic denaturation of collagen I unwinds the collagen structure, leading to exposure of RGD motifs, which may then interact with RGD-integrins. Since collagen I remodeling occurs via controlled biodegradation and synthesis of collagen generating equilibrium between native and denatured collagen I states (Taubenberger et al., 2010), it would be important in future studies to assess whether denatured collagen I with exposed RGD-binding motifs could influence BNP stimulated cGMP production. We also demonstrate that a second RGD-binding matrix protein collagen IV increased BNP stimulated cGMP, while a biologically inert, non-RGD-binding substrate poly-L-lysine had no effect on BNP stimulated cGMP generation, supporting and extending our hypothesis that RGD containing collagens have a specificity in the interaction with NPR-A and BNP

activation of cGMP in CFs. This conclusion is further strengthened by the lack of effect of collagen I which lacks an RGD region in its native state.

It is interesting to speculate that the ECM milieu, via RGD in FN binding to RGD-binding Arg-Arg-X (DDX) domains in the NPR-A may pull open the receptor-binding groove, inducing conformational changes which favor BNP binding and receptor activation. These results are similar to Cowles et al. (2000), who also reported that different anchorage proteins generated differential cell responses, although not involving BNP or CFs. Specifically, this group reported that signaling and proliferation of bone osteoblasts was highest when the cells were plated on FN compared to collagen I, where the increase was less, while poly-L-lysine showed no change in signaling or proliferation. Thus, the most significant enhancement of cellular activity, including cGMP by BNP, occurs with FN. Since collagen IV is part of the normal myocardial matrix and is not significantly different in the fibrotic heart (Peng et al., 2006; Khoshnoodi et al., 2008), while FN is increased in pathologic fibrosis, it is interesting to speculate that the changes in BNP/NPR-A/cGMP signaling to FN may be part of the mechanism of the anti-fibrotic response by BNP specifically to enhance opposing anti-fibrotic properties such as inhibition of CF proliferation and/or collagen production.

CFs compose 90% of the non-myocyte cells in the myocardium and play a major role in the regulation of the ECM (Gudi et al., 1998). They produce collagens type I, III, and IV as well as FN (Gudi et al., 1998). In addition, they express a large repertoire of integrin receptor subtypes, lacking $\alpha 6$ and $\alpha 7$ as CFs have no laminin-containing basement membrane, but uniquely expressing αv and $\alpha 2$ integrins which are absent on CMs (Ross and Borg, 2001). To begin to distinguish between NPR-A and integrin effects, we tested whether antibody blockade to specific integrin receptor sub-types that are expressed on CFs and bind to FN would alter BNP-induced cGMP production when plated on FN-coated plates. Specifically, we examined CF integrin subtypes which were known to block cell adhesion to FN or to ECM proteins including anti-human FNCB, anti-human integrin $\beta 1$, anti-human integrin $\beta 3$, and human anti-integrin $\alpha v\beta 3$. Blockade of FNCB, integrin $\beta 1$ and integrin $\beta 3$ had little effect. Importantly and in contrast, we observed that integrin $\alpha v\beta 3$ blockade induced a greater cGMP production by BNP than FN alone. Further, this augmented action of blockade of integrin $\alpha v\beta 3$ on cGMP production in CFs occurred even in the absence of FN plating and could be further augmented by co-incubation of both the integrin $\alpha v\beta 3$ antibody and our RGD peptide. These findings support the conclusion that integrin receptor blockade, such as integrin $\alpha v\beta 3$ here, and in contrast to $\beta 1$ and $\beta 3$, alters BNP-induced cGMP in different directions in CFs leading us to speculate that BNP/NPR-A/cGMP and integrin receptor subtypes may be counter-regulatory partners in mechanisms of fibrosis. We further conclude that this action of the integrin $\alpha v\beta 3$ receptor is independent of FN. These are the first studies in CFs to demonstrate that the activity of another receptor other than the NP receptors may influence cGMP production induced by BNP.

Our studies have physiological, pathophysiological, and therapeutic implications. As collagens are produced and secreted by fibroblasts and BNP is also produced and released from this cell type, there may exist a fine feedback mechanism whereby collagens influence BNP-regulated cGMP mechanisms of fibroblast proliferation and collagen production. In pathologic states associated with cardiac fibrosis, the endogenous BNP/cGMP system in CFs may be insufficient to adequately control the ECM and this could lead to excessive activation of integrin receptors such as $\alpha v\beta 3$. This would suggest that supplemental BNP, small NPR-A RGD mini-peptides as we used in the current studies or blockade of selective integrin receptors could serve as therapeutic strategies in targeting pathologic fibrosis in the heart.

In conclusion, ECM proteins, specifically collagen IV and FN, demonstrate crosstalk with the NPR-A receptor in the control of the second messenger cGMP when activated by the cardiac hormone BNP. Our current findings also demonstrate specific integrin receptor subtypes, here $\alpha_v\beta_3$, also serve as a counter-regulatory mechanism to BNP cGMP activation in CFs which to date has not been reported. These studies thus advance our understanding of the biology of CFs and regulation of the generation of cGMP by BNP, the ECM and integrins.

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Abbreviations

BNP	brain natriuretic peptide
NPR-A	natriuretic peptide receptor A
FN	fibronectin
ECM	extracellular matrix
CF	cardiac fibroblast
CMC	cardiomyocyte
RGD	Arg-Gly-Asp
DDX	Asp-Asp-any aa
cGMP	3'5' cyclic monophosphate guanosine

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Fig. 1. Cyclic GMP response in human cardiac fibroblasts to BNP with fibronectin, collagen I, collagen IV, or poly-L-lysine coated plates. Fibroblasts were grown on uncoated or matrix-coated plates and treated with 10^{-6} M BNP. Values are from six experiments and values are the mean of three samples per treatment.

Fig. 2. Cyclic GMP response in human cardiac fibroblasts to BNP with and without integrin receptor subtype antibody blockade. Fibroblasts were grown on uncoated or fibronectin coated plates and treated with 10^{-6} M BNP in the presence or absence of 1:100 dilution of integrin antibodies. Values are from four experiments and values are the mean of three samples per treatment.

Fig. 3.

A cyclic GMP generation in human cardiac fibroblasts to BNP with and without integrin $\alpha\text{v}\beta 3$ antibody blockade on uncoated or FN-coated plates. Fibroblasts were grown on uncoated or fibronectin coated plates and treated with 10^{-6} M BNP in the presence or absence of 1:100, 1:300, or 1:900 dilution of integrin $\alpha\text{v}\beta 3$ antibody. Values are from four experiments and values are the mean of three samples per treatment.

Fig. 4.

Cyclic GMP generation in human cardiac fibroblasts to BNP with and without integrin $\alpha v \beta 3$ antibody blockade and NPR-A RGD peptide. Fibroblasts were grown on uncoated plates and treated with 10^{-6} M BNP in the presence or absence of 1:100 dilution of integrin $\alpha v \beta 3$ antibody with or without NPR-A RGD peptide. Values are from four experiments and values are the mean of three samples per treatment.