Atrial natriuretic peptide suppresses endothelin gene expression and proliferation in cardiac fibroblasts through a GATA4-dependent mechanism

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Aims Atrial natriuretic peptide (ANP) is a hormone that has both antihypertrophic and antifibrotic properties in the heart. We hypothesized that myocyte-derived ANP inhibits endothelin (ET) gene expression in fibroblasts.

Methods and results We have investigated the mechanism(s) involved in the antiproliferative effect of ANP on cardiac fibroblasts in a cell culture model. We found that cardiac myocytes inhibited DNA synthesis in co-cultured cardiac fibroblasts as did treatment with the ET-1 antagonist BQ610. The effect of co-culture was reversed by antibody directed against ANP or the ANP receptor antagonist HS-142-1. ANP inhibited the expression of the ET-1 gene and ET-1 gene promoter activity in cultured fibroblasts. The site of the inhibition was localized to a GATA-binding site positioned between –132 and –135 upstream from the transcription start site. GATA4 expression was demonstrated in cardiac fibroblasts, GATA4 bound the ET-1 promoter both in vitro and in vivo, and siRNA-mediated knockdown of GATA4 inhibited ET-1 expression. ET-1 treatment resulted in increased levels of phospho-serine105 GATA4 in cardiac fibroblasts and this induction was partially suppressed by co-treatment with ANP.

Conclusion Collectively, these findings suggest that locally produced ET-1 serves as an autocrine stimulator of fibroblast proliferation, that ANP produced in neighbouring myocytes serves as a paracrine inhibitor of this proliferation, and that the latter effect operates through a reduction in GATA4 phosphorylation and coincident reduction in GATA4-dependent transcriptional activity.

KEYWORDS Cardiac fibroblasts; Atrial natriuretic peptide; Endothelin; GATA4

1. Introduction

The development of cardiac hypertrophy and subsequent progression to heart failure are accompanied by increased myocyte size and expansion of the fibroblast population in the interstitial compartment of the heart. The latter is associated with remodelling of the extracellular matrix and increased interstitial and perivascular fibrosis. In aggregate, these changes are linked to both diastolic and systolic dysfunction in the heart. Therefore, a better understanding of the molecular factors involved in signalling these changes and the regulatory controls that govern them may lead to improvement in therapeutic strategies for addressing the progression from hypertrophy to heart failure clinically.

A variety of growth factors and vasoactive peptides have been implicated as playing a role both in promoting myocyte growth and in fostering the fibroblast proliferation and matrix deposition that is seen in the heart coincident with hypertrophy. Transforming growth factor-beta (TGF-β), angiotensin II (AII), and endothelin (ET), among others, have been linked to myocyte growth, fibroblast proliferation, and the development of interstitial cardiac fibrosis in various animal models and clinical disease in humans.

Antagonists of myocyte hypertrophy and the pro-fibrotic process have also been identified. Most notable in this group are the natriuretic peptides which are produced locally in the heart. Atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) are each produced within the cardiac myocyte. Both bind with near equivalent affinity to the type A natriuretic peptide receptor (NPR-A). C-type natriuretic peptide (CNP) appears to be produced primarily in the fibroblasts of the heart. It serves as the primary ligand for the type B natriuretic peptide receptor.

Exogenous ANP, BNP, and CNP have each been shown to antagonize the pro-hypertrophic and/or pro-fibrotic actions of TGF-β, AII, and/or ET-1 in cardiac myocytes and fibroblasts, either in vivo or in vitro. Germline deletion of the BNP gene or that of its cognate receptor NPR-A.

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is associated with increased interstitial fibrosis, procollagen synthesis and, in the case of the NPR-A knockout, increased myocyte hypertrophy. These effects are exaggerated in the presence of a hypertrophic stimulus (e.g. thoracic aorta constriction).

BNP exerts a broad-based antagonism of TGF-β-dependent pro-fibrotic activity in cultured cardiac fibroblasts. Recent studies suggest that ANP, and by inference BNP, may prevent TGF-β-dependent phosphorylation of Ser109 and Thr388 in the SMAD3 molecule, an effector of TGF-β, thereby preventing SMAD3 nuclear translocation and activation of downstream TGF-β activity. Aside from this example, relatively little is known about the mechanism(s) underlying the NP-dependent inhibition of agonist-stimulated fibroblast proliferation or synthetic activity.

In the present studies, we reasoned that ANP might act at least in part through inhibition of ET-1 gene expression. We demonstrate that both exogenous ANP and endogenous peptide(s) released from the cardiac myocyte inhibit growth of fibroblasts cultured from neonatal rat hearts. It does so in association with reduced phosphorylation of the transcription factor GATA-4, inhibition of GATA-4-dependent ET-1 association with reduced phosphorylation of the transcription factor GATA-4, inhibition of GATA-4-dependent ET-1 gene promoter activity, and subsequent reduction of endogenous ET-1 gene expression in these cultures.

2. Methods

2.1 Materials

ET-1 was purchased from American Peptide (Sunnyvale, CA, USA); ANP was from Phoenix Pharmaceuticals (Burlingame, CA, USA). Antibodies directed against GATA4, GATA2, Egr-1, phospho-ERK2, and ERK2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The phospho-GATA4 antibody (ab5245) was obtained from Abcam Inc. (Cambridge, MA, USA). Anti-ANP antibody was from Phoenix Pharmaceuticals. Isobutylmethylxanthine (IBMX) and BQ610 were purchased from Sigma-Aldrich (St Louis, MO, USA). HS-142-1 was a gift from Kyowa Pharmaceuticals (Princeton, NJ, USA). The GATA4 sRNA construct was purchased from Openbiosystems (Huntsville, AL, USA). The −1315 rat (r)ET-1-luciferase reporter plasmid was generously provided by Paul et al. Mutations in the GATA and AP1 elements were introduced in the context of the −1315 rET-1 luciferase reporter plasmid by site-directed mutagenesis using the Quickchange kit (Stratagene, La Jolla, CA, USA).

2.2 Cell culture

Ventricular myocytes and fibroblasts were prepared from 1- to 2-day-old neonatal Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) as described previously. All experiments were approved by the Institutional Animal Care and Use Committee at University of California at San Francisco (UCSF) and comply with the guides for the care and use of laboratory animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Fibroblasts were used after one passage in culture. Both cell types were maintained in Dulbecco’s modified Eagle’s medium (DMEM) H-21 supplemented with 10% enriched calf serum (ECS). The cells were changed to serum-free medium before initiation of each experiment. Co-cultures were plated in a 12-well plate with Corning Transwell-Clear Permeable Supports.

2.3 3H-thymidine incorporation

After serum starvation for 12 h, cells were treated with 10−7 M ET-1 for 36 h. During the final 12 h, they were incubated with 3H-thymidine (4 µCi/mL) in thymidine-free Eagle’s minimal essential medium. 3H-thymidine incorporation assay was carried out as described previously.

2.4 Transfection and luciferase assay

Cardiac fibroblasts were transiently co-transfected with a −1315 rET-1 luciferase reporter, or the corresponding GATA and AP1 mutant constructs, and Renilla luciferase by electroporation as reported previously. Twenty-four hours following transfection, cells were incubated with vehicle or ANP (10−7 M) for an additional 24 h. At that time point, the cells were collected and lysed. Luciferase activity was measured using the Dual-Luciferase kit (Promega, Madison, WI, USA). ET-1 promoter-dependent Firefly luciferase activity was normalized for Renilla luciferase activity.

2.5 Total RNA isolation and quantitative PCR

Total RNA was isolated from neonatal rat cardiac fibroblasts with the RNeasy kit (Qiagen, Germany) and reverse transcribed into cDNA. Real-time PCR was carried out with rat pre-proET-1 (Rn00561129_m1) and GAPDH (Rn99999916_sl) Taqman primers (Applied Biosystems, Foster City, CA, USA).

2.6 Lentiviral preparation and infection

Lentivirus was prepared as described previously. Virus was handled according to established bio-safety protocols. Following serum deprivation, lentivirus was directly applied to the media and cells were incubated for an additional 24 h prior to treatment with vehicle or ANP (10−7 M) for 1 h.
2.7 Immunoblotting

Following initial isolation, fibroblasts were changed from medium containing 10% ECS to serum-free media for 18 h. At that point, vehicle, ANP or ET-1 was added to the media. Cells were cultured for another 24–48 h before total cell or nuclear lysates were prepared as described previously.\(^{22}\) Total protein was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to membranes. The membranes were probed with an antibody directed against GATA4, phospho-GATA4, ERK2, or phospho-ERK2. Blots were incubated with horseradish peroxidase-conjugated secondary antibodies and visualized by chemiluminescence (SuperSignal West Femto, Pierce Protein Research Products, Rockford, IL, USA).

2.8 Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSAs) were performed with isolated cardiac fibroblast nuclear extracts and \(^{32}\)P-labelled oligonucleotide harbouring the candidate GATA4-binding sequence as described previously.\(^{22}\) Nuclear extracts were incubated in binding reaction buffer (10 mM HEPES, pH 7.9, 50 mM KCl, 0.2 mM EDTA,
2.5 mM dithiothreitol, 10% glycerol, and 0.05% Nonidet P-40) containing 0.5 mg of poly(dI-dC) and 32P-end-labelled double-stranded wild-type ET-1 (5'-CCTCTAGAGCCGGGTCTTATCTCCGGCTGCACGTTG) or the GATA mutant (5'-CCTCTAGAGCCGGGTCTTcCTCCGGCTGCACGTTG) oligonucleotide on ice for 30 min. Mutated bases are shown in bold lower case. All samples were resolved on 4% non-denaturing polyacrylamide gels. Gels were dried and exposed to X-ray film.

2.9 Immunofluorescence

Fibroblasts were maintained in DMEM H-21 supplemented with 10% foetal bovine serum, prior to fixation with 4% paraformaldehyde in PBS. Slides were subjected to immunocytochemistry using goat polyclonal anti-mouse GATA4 (sc-1237, Santa Cruz Biotechnology) (1:100 diluted), mouse monoclonal anti-mouse GATA2 IgG (sc-267, Santa Cruz Biotechnology) (1:100 diluted), or mouse monoclonal anti-vimentin IgG (C 9080, Sigma-Aldrich) (1:150 diluted). Anti-mouse Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) and anti-goat Cy3 (Invitrogen) secondary antibodies were used. Samples were then analysed by light and contrast microscopy (Leica DMRXA microscope).

2.10 Chromatin immunoprecipitation assay

Cells were cultured in serum-free media and treated with ANP and/or ET-1 for an additional 24 h. The DNA-IP assays were performed using a modification of published methodology. Briefly, after treatment, cells were fixed with 1% formaldehyde for 15 min at 37°C, neutralized with 0.125 M glycine for 5 min at room temperature, washed, lysed, and sonicated. The supernatant was pre-incubated with protein G sepharose beads, 2 μg salmon sperm DNA, 100 mg/mL bovine serum albumin, and shaken at 4°C overnight. At that point, the supernatant was divided, either anti-GATA4 antibody or normal rabbit IgG was added, and the incubation was continued at 4°C overnight. Immunoprecipitates were collected, then sequentially washed as described. Bound material was eluted with freshly made elution buffer (1% SDS and 0.1 M NaHCO3). Cross-linking was reversed by heating the elutes at 65°C overnight. DNA was extracted and PCR was performed with a primer pair that spans the rET-1 gene transcription start site (5′ primer: 5′-TTCATTCAATGGGGTGACT and 3′ primer: 5′-GGGGTAAACAGCTCCGACTT).

2.11 Statistical analysis

Data was analysed by one-way ANOVA using the Student-Newman-Keuls post hoc test to assess significance.

3. Results

3.1 ANP inhibits fibroblast proliferation

ANP inhibited 3H-thymidine incorporation, a measure of DNA synthesis and cell proliferation, in fibroblasts cultured from neonatal rat hearts, similar to what has been reported previously (Figure 1A). The level of inhibition (~40–45%) was increased slightly by inclusion of IBMX, a non-selective phosphodiesterase inhibitor, in the incubation medium, suggesting that the ANP-dependent second messenger cGMP is mediating this inhibition. 3H-thymidine incorporation was also reduced by the type A ET receptor antagonist BQ-610. Interestingly, this reduction was not additive with that produced by ANP.

We next tested the ability of endogenously produced myocyte factors to regulate DNA synthesis in neighbouring fibroblast cells. We co-cultured fibroblasts with cardiac...
myocytes, harvested from the same neonatal hearts, on surfaces separated by a semi-permeable membrane, then pulsed the fibroblasts with $^3$H-thymidine to assess DNA synthesis. As shown in Figure 1B, co-culture with cardiac myocytes reduced $^3$H-thymidine incorporation in the fibroblast population. Inclusion of BQ610 in the incubation led to reduction in DNA synthesis in the control fibroblast cultures, supporting the findings above, but did not further amplify the inhibition seen in the co-cultures. Once again, the addition of IBMX resulted in a modest increase in the level of inhibition in the fibroblast cultures and in the fibroblast/myocyte co-cultures. We postulated that the factor responsible for this inhibition was ANP produced in and secreted from the cardiac myocytes. To explore this possibility, we included either an antibody directed against ANP or the NPR-A inhibitor HS-142-1 in the medium bathing the fibroblasts. While neither the antibody nor HS-142-1 affected basal DNA synthesis in the fibroblast cultures (Figure 1B), arguing against significant natriuretic peptide expression by these non-myocytes, each completely reversed the inhibition seen in the co-cultures. Thus, it appears that ANP released from cardiac myocytes has the ability to suppress DNA synthesis and, inferentially, cell proliferation, in the neighbouring fibroblast population.

3.2 ANP inhibits ET-1 gene expression

ET-1 gene expression has been identified in both myocyte and fibroblast populations in the heart. The findings presented in Figure 1 argue that endogenous fibroblast-derived ET-1 is capable of auto-activating DNA synthesis in these cells. The fact that ANP and BQ610 are not additive in suppressing $^3$H-thymidine incorporation raises the intriguing possibility that ET-1 and ANP operate over a shared pathway(s) in regulating fibroblast proliferation. This suggested to us that myocyte-derived ANP might exert its inhibitory activity through suppression of ET-1 gene expression in cardiac fibroblasts. To test this hypothesis, we introduced a reporter construct harbouring 1315 bp of rET gene promoter sequence (−1315 rET-1-Luc) into cultured fibroblasts, then placed them in co-culture with neonatal cardiac myocytes. As shown in Figure 2A, the presence of the myocytes in the co-cultures resulted in ~40% inhibition of ET-1 gene promoter activity. This was completely reversed, in dose-dependent fashion, by inclusion of HS-142-1 in the incubation medium. Independent experiments confirmed that exogenous ANP was capable of reducing ET-1 mRNA transcript levels in fibroblasts (Figure 2B) and suppressing ET-1 promoter activity (Figure 2C). Collectively, these data imply that fibroblast-derived ET-1 promotes DNA synthesis in an autocrine fashion in these cultures. ANP produced from neighbouring cardiac myocytes has an inhibitory effect on fibroblast DNA synthesis and this inhibition results, at least in part, from ANP-dependent suppression of ET-1 gene transcription in the fibroblast population.

3.3 ANP inhibition of ET-1 expression is mediated by a GATA element

We next asked how ANP might exert its effects on the ET-1 gene promoter. Deletion data presented in Figure 2C indicated that the ANP-responsive element(s) responsible for the inhibition of promoter activity was positioned between −421 and −81 bp relative to the transcription start site. We identified two regulatory elements in the proximal rat gene promoter, a GATA element at position −132 bp and AP1 at position −102 bp, relative to the transcription start site, that have been shown previously to contribute to basal transcriptional activity of this gene. Each of these was mutated in the context of −1315 rET-1-Luc and introduced into cardiac fibroblasts. Twenty-four hours after transfection, cultures were treated with ANP (10$^{-7}$ M) for an additional 24 h, cells were harvested, and cell extracts were generated for luciferase analysis. As shown, mutation of the AP-1 site led to a decrease in basal promoter activity but did not impact the ANP-dependent inhibition.
at baseline; however, the combination of ET-1 plus ANP resulted in a significant reduction in DNA binding relative to the control cultures treated with ET-1 alone. To determine the nature of the protein associated with the GATA site, we pre-incubated the control extracts with antibody directed against GATA4, GATA2, or an unrelated protein Egr-1. Only the antibody against GATA4 resulted in a super-shift of the complex to a higher position in the gel. Interaction of the probe with GATA4 appeared to require the GATA site identified above since mutation of this site completely eliminated the DNA–protein interaction (Figure 3A). The interaction of GATA4 with the ET-1 gene promoter was confirmed using chromatin immunoprecipitation analysis (Figure 3B), demonstrating that the interaction takes place within the context of the intact cell. In this setting, pre-treatment with ET-1 led to a significant increase in GATA4 binding. Although ANP alone had little effect on binding activity (statistically not significant), it had a dramatic effect in reducing the binding induced by ET-1 (Figure 3C).

3.5 GATA4 is present in cardiac fibroblasts
Although GATA4 has been identified in cardiac myocytes, where it has been shown to play a pivotal role in the control of gene expression associated with myocyte hypertrophy, it has not been reported to be present in fibroblasts of the heart nor to be an important regulator of fibroblast gene expression. To provide some assurance that the putative GATA4 activity did not result from the presence of contaminating cardiac myocytes in our cultures, we carried out immunocytochemistry for the GATA4 protein. As shown in Figure 4, the preponderance of cell nuclei in our fibroblast cultures stained for GATA4. Staining of the same cultures with anti-vimentin antibody (a cellular protein which is largely confined to fibroblasts) also stained a majority of the cells on the plate. The merged image clearly demonstrates co-localization of GATA4 and vimentin in the same cells. A similar approach using antibody directed against GATA2 failed to identify any GATA2-expressing cells.

3.6 GATA4 knockdown inhibits ET-1 gene expression
To further demonstrate the role of GATA4 in ET-1 gene expression, we infected cardiac fibroblasts with lentivirus containing a GATA4 siRNA construct. GATA4 knockdown was confirmed by western blot analysis (Figure 5A and B). Lentivirus-expressing green fluorescent protein (GFP) had no effect on GATA4 expression levels. However, in the presence of the GATA4 siRNA, expression of ET-1 was reduced to a level similar to that seen in the presence of ANP (Figure 5C). Thus, reduction of GATA4 levels parallels reductions in ET-1 gene expression and suggests that ANP inhibits ET-1 gene expression through a GATA4-dependent mechanism.

3.7 ANP inhibits ET-mediated ERK activation and GATA4 phosphorylation
Next, we attempted to probe the mechanism whereby ANP might antagonize GATA4-dependent transcriptional activity. GATA4 in cardiac myocytes is known to be activated by a
mitogen-activated protein kinase-dependent phosphorylation of Ser\textsuperscript{105}.\textsuperscript{23,31} This phosphorylation leads both to enhanced DNA binding and to increased transcriptional activity of the targeted gene.\textsuperscript{31} ET-1 treatment of cardiac fibroblasts led to a marked increase in levels of phospho-GATA4, an effect that was completely blocked by the MEK inhibitor U0126 (Figure 6A and B). Cardiac fibroblasts were serum starved and then treated with $10^{-7}$ M ANP or vehicle followed by treatment with ET-1 ($10^{-7}$ M). (E) Cells were pre-treated with $10^{-7}$ M ANP or vehicle for 10 min, followed by 10 min treatment with $10^{-7}$ M ET. Nuclear extracts were prepared from cells and phospho-GATA4 protein was detected by western blot analysis. *P < 0.05.

Figure 6  ANP inhibits ET-induced phosphorylation of ERK2 and GATA4. Representative experiments (A, C, and E) and pooled data (B, D, and F) from three experiments are shown. (A) Cells were maintained in serum-free media for 18 h, then pre-treated with $10^{-5}$ M U0126 or vehicle for 1 h followed by 10 min treatment with $10^{-7}$ M ET-1. Nuclear extracts were prepared from cells and phospho-GATA4 protein was detected by western blot analysis. (C) ERK2 activation was assessed by western blot with an anti-phospho-ERK2 antibody. Cardiac fibroblasts were serum starved and then treated with $10^{-7}$ M ANP or vehicle followed by treatment with ET-1 ($10^{-7}$ M). (E) Cells were pre-treated with $10^{-7}$ M ANP or vehicle for 10 min, followed by 10 min treatment with $10^{-7}$ M ET. Nuclear extracts were prepared from cells and phospho-GATA4 protein was detected by western blot analysis. *P < 0.05.

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4. Discussion
The major points to be drawn from the current study include: (i) endogenously produced myocyte ANP plays a pivotal role in down-regulating proliferative activity in the neighbouring fibroblast compartment, (ii) this down-regulation relies, at least in part, on NP-dependent inhibition of ET-1 gene expression in the fibroblasts, (iii) this inhibition involves suppression of ET-1 gene promoter activity through a proximal GATA factor-binding site in that promoter, (iv) GATA4, a transcription factor more typically associated with the cardiac myocyte, is present in cardiac fibroblasts and appears to associate with the GATA-binding element on the ET-1 gene promoter both in vitro and in the context of the intact cell, and (v) ANP-dependent
Cardiac fibroblasts to activate hypertrophic growth in the heart, but these studies have focused on the capacity of paracrine interactions between myocytes and fibroblasts in the heart. PDGF and IGF1, and inflammation (COX2 and IL6). (e.g. collagen 1 and fibronectin), myofibroblast conversion (e.g. α-smooth muscle actin), fibroblast proliferation (e.g. PDGFA and IGF1), and inflammation (COX2 and IL6).

A recent study from Li et al., provides convincing evidence that ANP, as well as its second messenger cyclic GMP, inhibits TGF-β1-induced phosphorylation of SMAD3 at specific phospho-accepting amino acid residues (i.e. Ser105 and Thr188). This prevents nuclear translocation of SMAD3 and activation of downstream events like myofibroblast transformation and proliferation, and synthesis of extracellular matrix proteins. The current study suggests a separate mechanism for ANP-dependent inhibition of ET-1's pro-fibrotic activity. Findings presented in Figure 6 indicate that ANP reduces levels of phospho-ERK2 and subsequent phosphorylation of Ser105 in the GATA4 protein, an effect that would be predicted to reduce activity of this transcription factor. Thus, post-translational modification of transcription factors that negatively affects their transcriptional activity represents a recurrent theme in describing ANP's anti-proliferative activity in the cardiac fibroblast.

Although ET-1 has been linked to pro-fibrotic activity in cardiac fibroblasts, antagonism of ET-1 activity by NPs in these fibroblasts has received only limited attention. ANP has been shown to inhibit ET-1 gene expression in fibroblasts and in cultured endothelial cells. However, the mechanism details underlying this inhibition have not been elucidated. ET-dependent activation of the rat BNP gene promoter in cardiac myocytes is critically dependent on the presence of two tandemly arrayed GATA4-binding elements positioned between −70 and −100 bp in that promoter. It is noteworthy that a similar GATA4-binding element, identified here, appears to be responsible for mediating the NP-dependent inhibition of the ET-1 gene promoter in cardiac fibroblasts, suggesting a unifying mechanism in closing this local autocrine-paracrine loop (Figure 7).

In conclusion, we have shown that endogenous ANP production in cardiac myocytes exerts a negative regulatory influence on proliferation of neighbouring fibroblasts by suppressing the expression of ET, an autocrine stimulator of fibroblast growth. This inhibition occurs in parallel with reduced phosphorylation and, inferentially, activity of the transcription factor GATA4, a key regulator of ET-1 gene transcription.

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