17β-Estradiol Antagonizes Cardiomyocyte Hypertrophy by Autocrine/Paracrine Stimulation of a Guanylyl Cyclase A Receptor–Cyclic Guanosine Monophosphate–Dependent Protein Kinase Pathway

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Background—Significant gender-related differences exist in the development of left ventricular hypertrophy (LVH). In addition, administration of 17β-estradiol (E_2) to ovariectomized female mice attenuates the development of LVH, demonstrating an antagonistic role for E_2 in this process, although no molecular mechanism has been proposed for this phenomenon.

Methods and Results—E_2 attenuated phenylephrine and endothelin-1 induced hypertrophy in neonatal cardiomyocytes, and E_2 directly induced atrial natriuretic factor (ANF) expression as assessed by Northern blot, immunocytochemical analyses, and transient transfection assays using ANF promoter deletion fragments. Both the antihypertrophic effects and ANF induction could be blocked by the estrogen receptor antagonist ICI 182,780, which demonstrates a genomic, estrogen receptor–dependent pathway. To mimic E_2-induced autocrine/paracrine effects through stimulation of the guanylyl cyclase A receptor (ANF receptor), cardiomyocytes were stimulated with phenylephrine or endothelin-1 in the presence of exogenous ANF or 8-bromo–cyclic guanosine monophosphate (cGMP), both of which attenuated agonist-induced hypertrophy. Both estrogen and ANF increased cGMP activity. The antihypertrophic effect of ANF could be reduced with extracellular ANF antibodies in a dose-dependent manner. cGMP-dependent protein kinase mediates the antihypertrophic effects of E_2, so cardiomyocytes were agonist stimulated in the presence of the cGMP-dependent protein kinase blocker KT-5823. KT-5823 not only reversed the antihypertrophic properties of E_2, ANF, or 8-bromo-cGMP, but also evoked potentiation of hypertrophy.

Conclusions—E_2-mediated induction of ANF in cardiac hypertrophy contributes to its antagonistic effects in LVH. **(*Circulation*. 2004;109:269-276.)**

Key Words: hypertrophy ■ remodeling ■ cells ■ genes

The mammalian heart elicits hypertrophy in response to stimuli that elevate wall stress in an attempt to decrease wall tension.[1] Cardiomyocyte hypertrophy is characterized by an increase in cellular volume and enhanced sarcomeric organization of individual myocytes.[2] The molecular response of ventricular myocytes to left ventricular hypertrophy (LVH) involves the reinduction of genes transiently expressed during embryogenesis[2] and is initiated by complex cascades of cytoplasmic signaling events.[3]

Sex hormones such as estrogen have properties that are of potential benefit in inhibiting the progression of cardiac disease. The incidence and severity of cardiovascular disease, including LVH, in premenopausal women is lower than in men of comparable age, even after correction for various risk factors.[4] However, the molecular effects of estrogen on ventricular cardiomyocytes, which may account for this clinical observation, are less well understood. Cardiomyocytes express functional estrogen receptors (ERs), and estrogen treatment modulates the expression of cardiac-specific genes.[5] Recently, we demonstrated that female ovariectomized mice develop a more robust LVH response in a model of pressure overload than ovariectomized mice with replacement of physiological levels of 17β-estradiol. In particular, more pronounced ventricular expression of atrial natriuretic factor (ANF) in the banded, estrogen-supplemented group than in vehicle-treated, banded animals was noticeable.[6]

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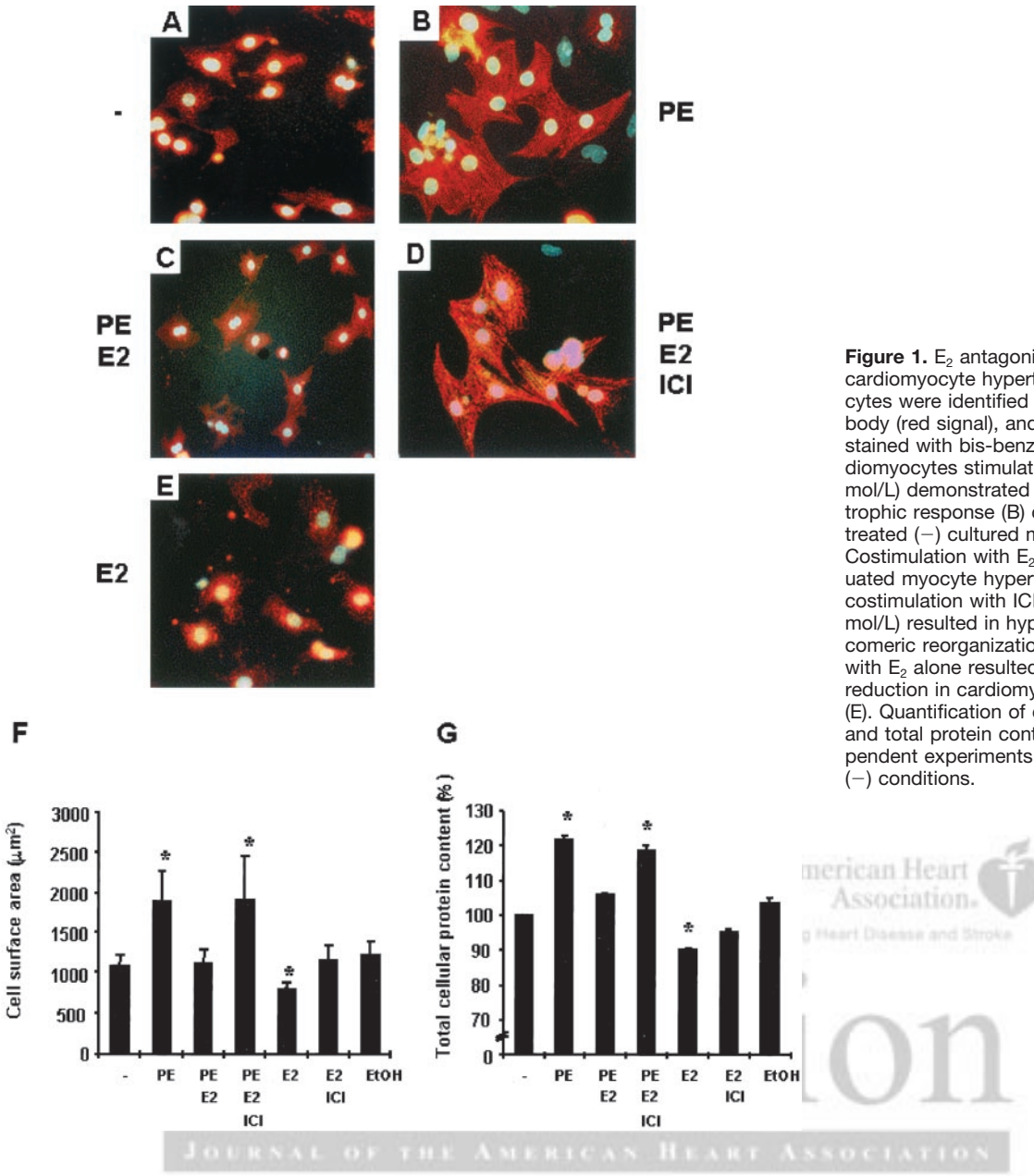

Figure 1. E_2 antagonizes PE-induced cardiomyocyte hypertrophy. Cardiomyocytes were identified with α-actinin antibody (red signal), and nuclei were stained with bis-benzamide (white). Cardiomyocytes stimulated with PE (10^{-5} mol/L) demonstrated a significant hypertrophic response (B) compared with nontreated (−) cultured myocytes (A). Costimulation with E_2 (10^{-9} mol/L) attenuated myocyte hypertrophy (C), whereas costimulation with ICI 182,780 (10^{-5} mol/L) resulted in hypertrophic and sarcomeric reorganization (D). Stimulation with E_2 alone resulted in a detectable reduction in cardiomyocyte surface area (E). Quantification of cell-surface area (F) and total protein content (G) of 3 independent experiments. *$P<0.05$ vs control (−) conditions.

ANF is a peptide hormone that under physiological conditions displays a restricted atrial expression pattern in the adult heart.[7] Increasing evidence favors the notion that ANF may function as a local endocrine antagonist of LVH.[8] In support of this notion, ANF administration to cultured cardiomyocytes has been associated with growth-limiting effects.[9]

Here, we studied the relationship between estrogen-mediated antagonism of hypertrophy, ANF expression, and guanylyl cyclase A (GC-A) receptor signaling in cultured cardiomyocytes. Estrogen exerted profound antihypertrophic effects on ventricular myocytes, and this finding was accompanied by a direct stimulation of ANF transcription. Both ANF and 8-bromo–cyclic guanosine monophosphate (cGMP) exhibited similar antihypertrophic effects in a dose-dependent manner, suggesting autocrine/paracrine stimulation of GC-A receptor signaling after estrogen administration.

Methods

Materials

17β-Estradiol (E_2), 17α-estradiol, hydroxytamoxifen, phenylephrine (PE), endothelin (ET-1), 8-bromo-cGMP, ANF, KT-5823, antiserum to sarcomeric α-actinin (EA-53), tetramethylrhodamine β-isothiocyanate (TRITC)–conjugated goat anti-mouse antibodies, and fluorescein isothiocyanate (FITC) goat anti-rabbit antibodies were purchased from (Sigma Aldrich). ICI 182,780 was a kind gift from Dr A. Wakeling (AstraZeneca, Wilmington, Del), and monoclonal and polyclonal anti-ANF antibodies were obtained from Peninsula Laboratories. Vectashield was purchased from Vector Laboratories. Animals were handled according to the guidelines of the animal welfare committee of the University of Maastricht. Primary cultures of 1- to 4-day-old Lewis neonatal rat ventricular myocytes (NRVMs) were obtained as described previously.[10]

Transfection Analysis: Luciferase

Human embryonic kidney (HEK293) cells were maintained in DMEM (Life Technologies) supplemented with 10% FBS and transiently transfected with pGL3 vectors containing ANF promoter

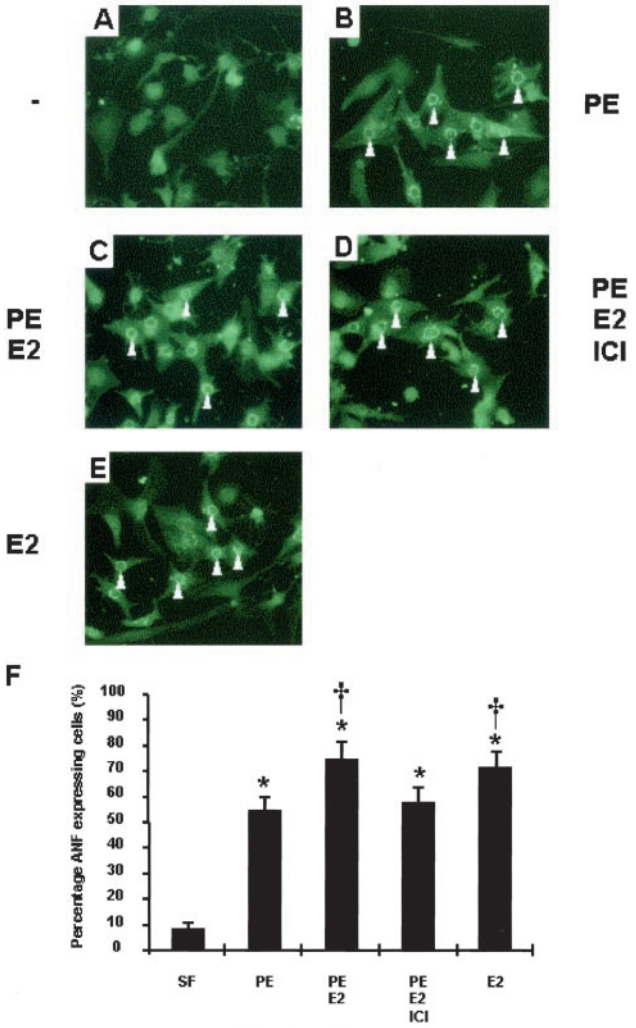

Figure 2. E_2 stimulates ANF mRNA expression. Cardiomyocytes were stained with ANF antibody (green). Cardiomyocytes were stimulated with PE (10^{-5} mol/L), with or without presence of E_2 (10^{-9} mol/L) and/or ICI 182,782 (10^{-5} mol/L), for 48 hours and immunostained with an antibody against ANF. Cells cultured under serum-free conditions (−) revealed no ANF protein expression (A), whereas PE (B) induced a typical perinuclear ANF protein expression (white arrows). Presence of E_2 (C) increased ANF protein expression, which was reduced in presence of ICI 182,780 (D). Stimulation of cardiomyocytes with E_2 alone resulted in an abundant ANF expression (E). Quantification of percent ANF-expressing cells (F) from 3 independent experiments. *$P<0.05$ vs control (−) conditions; †$P<0.05$ vs PE.

deletion fragments encompassing 700 bp (ANF-700 luc) or 150 bp (ANF-150 luc) of the proximal rat ANF promoter,[11] an estrogen receptor-α expression vector (HEG0, kind gift from Dr P. Chambon, Université Louis Pasteur/Collège de France, Illkirch, France), and SV40-βGal (Promega) using the lipid-based reagent Fugene 6 (Roche Molecular Biochemicals) in accordance with the manufacturer's protocol.[12] A set of 3 individual transfection experiments were performed and measured 3 times. NRVMs were treated with E_2 (10^{-9} mol/L) and/or ICI 182,782 (10^{-5} mol/L) for 48 hours and harvested as described earlier. Data are presented as relative luciferase activity based on the luciferase/galactosidase (Luc/Gal) ratio.

Northern Blot Analysis

Total RNA was isolated from cardiomyocytes with TRIzol reagent (Life Technologies). Northern blot hybridizations were performed by use of a modified protocol.[13] In brief, a 600-bp fragment of rat ANF cDNA or a 300-bp fragment of rat GAPDH cDNA was labeled with [^{32}P]dCTP (Dupont de Nemours, NV) using a random labeling kit (Life Technologies), added to the hybridization solution at 1×10^6 cpm/mL, and incubated overnight at 58°C. Stringent posthybridization wash conditions were used (0.1×SSC, 0.1% SDS at 58°C).

Immunocytochemistry

Cardiomyocytes were prepared for immunocytochemistry as described previously.[10] Morphological changes were documented with a Nikon Eclipse CFL60 Epifluorescence Microscope. Surface area measurements were performed on fixed cardiomyocytes using NIH image analysis software (Ascion). At least 100 NRVMs in 20 to 25 fields were examined in each experiment, and data are expressed as pooled averages of 3 independent experiments.

Biochemical Analysis

Lactate dehydrogenase (LDH) activity was measured. TUNEL assay was performed with the CardioTACS kit (Trevigen) according to the manufacturer's instructions on both stimulated and nonstimulated NRVMs.[14] NRVMs were treated with an ice-cold lysis buffer containing 0.5% Nonidet P40, 150 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 8.0, 0.5 mmol/L EDTA in the presence of a cocktail of proteinase inhibitors (2 μg/μL leupeptin, 10 μg/mL PMSF [Sigma]), and 2 μg/mL soybean trypsin inhibitor [Gibco BRL]). The amount of protein was estimated by the method of Bradford. ANF levels were measured by radioimmunoassay.

Statistical Analysis

Data are expressed as mean±SEM. For all statistical analyses, Instat 3.0 GraphPad software was used. Differences between experimental groups were evaluated for statistical significance by either 1-way ANOVA followed by Bonferroni's post hoc test or a Student's *t* test when appropriate. Values of $P<0.05$ were considered to be statistically significant.

Results

Estrogen Antagonizes Cardiomyocyte Hypertrophy

NRVMs were exposed to the hypertrophic agonist PE (10^{-5} mol/L) for 48 hours. PE induced a typical hypertrophic phenotype characterized by increased cell-surface area and enhanced sarcomeric organization compared with cells exposed to vehicle treatment (Figure 1, A and B). The presence of E_2 in a physiological concentration (10^{-9} mol/L) attenuated the PE-induced hypertrophic morphology (Figure 1C) substantially. Coadministration of the ER antagonist ICI 182,780 (10^{-5} mol/L) resulted in reappearance of the hypertrophic morphology and sarcomeric organization (Figure 1D). E_2 stimulation alone resulted in a reduction in cardiomyocyte size (Figure 1E). Similar results were obtained when the NRVMs were exposed to 10^{-7} ET-1 in the presence or absence of E_2 (data not shown).

PE administration showed an approximate increase of 69% in cell-surface area. In line with the immunocytochemical observations, the presence of E_2 reduced PE-induced increase in cell-surface area by 85% ($P<0.05$ versus PE alone). The effect of E_2 on PE-induced hypertrophy was fully reversed by coincubation with ICI 182,780 (Figure 1F). Similar findings were obtained for cardiomyocytes stimulated with ET-1 (data not shown).

To further demonstrate the antihypertrophic effect of E_2, the total cellular protein content of cardiomyocytes subjected

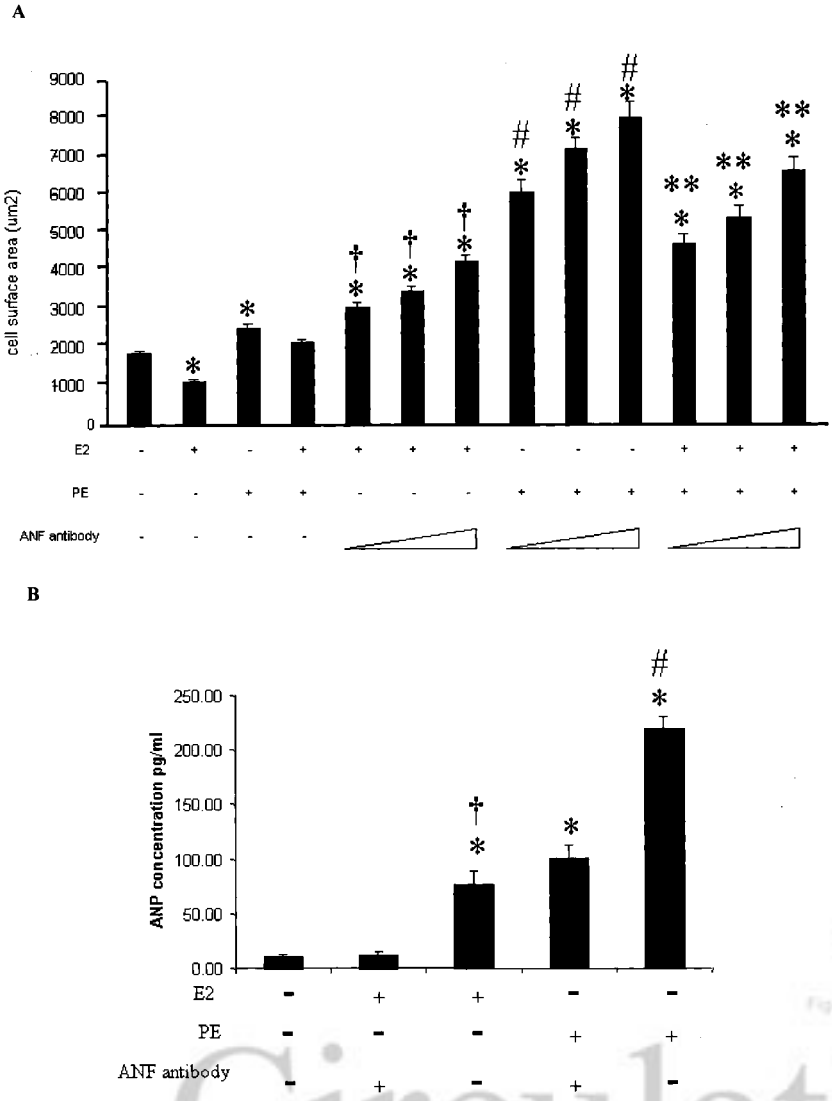

Figure 3. ANF antibody reverses antihypertrophic properties of estrogen in cardiomyocytes (A). *$P<0.05$ vs control (−) conditions, †$P<0.05$ vs E_2, #$P<0.05$ vs PE, and **$P<0.05$ vs PE/E_2. ANF antibody depleted ANF in culture medium (B). *$P<0.05$ vs control (−), †$P<0.05$ vs E_2 and ANF antibody, and #$P<0.05$ vs PE and ANF antibody.

to the different treatment conditions was determined. PE-induced hypertrophy was associated with a 21±2% increase in total cellular protein content ($P<0.05$ versus untreated cells; Figure 1G). E_2 stimulation prevented PE-induced increase in protein synthesis, whereas treatment with ICI 182,780 reversed the E_2-mediated antihypertrophic effects (Figure 1G). E_2 stimulation alone evoked a small but significant reduction in total cellular protein content, an effect abolished in the presence of ICI 182,780 (Figure 1G).

To exclude the potential of E_2-mediated cellular stress and toxicity in our experimental model, LDH activity as a marker of viability was determined in the culture medium. No difference in LDH was detected under any condition tested compared with untreated cardiomyocytes (data not shown). Furthermore, in a subset of experiments, E_2-treated cardiomyocytes were subjected to TUNEL staining, but no induction of cardiomyocyte apoptosis was evident (data not shown). Taken together, these results indicate that E_2 specifically antagonizes agonist-induced cardiomyocyte hypertrophy. Moreover, the effects were efficiently reversed by addition of the ER antagonist ICI 182,780, confirming that ER-dependent mechanisms are involved.

Estrogen Specifically Induces ANF Gene Expression

ANF immunoreactivity of cardiomyocytes was analyzed in the presence of PE and/or E_2. α-Adrenergic stimulation resulted in an increased intensity of perinuclear ANF staining in a larger number of cardiomyocytes compared with non-treated cardiomyocytes (Figure 2, A and B). Costimulation with PE and E_2, a condition that was associated with attenuation of all morphological aspects of myocyte hypertrophy (Figure 1C), did not diminish ANF immunoreactivity (Figure 2C), whereas the presence of ICI 182,780 did reduce ANF (Figure 2D). In fact, E_2 treatment of NRVMs in the absence of PE was sufficient to induce the typical perinuclear ANF staining (Figure 2E).

Quantification of ANF-positive cardiomyocytes supported the immunocytochemical observations. Only 8±2% of cardiomyocytes cultured under serum-free conditions displayed the characteristic perinuclear ANF staining pattern. In contrast, 54±4% of PE-stimulated cardiomyocytes revealed intense ANF immunoreactivity ($P<0.05$ versus serum-free; Figure 2F). Costimulation of PE and E_2 resulted in additional

induction of ANF immunoreactivity in a larger number of cells (73±9%, $P<0.05$ versus serum-free and PE; Figure 2F), whereas ICI 182,780 reduced this percentage back to the level of PE stimulation alone. Most strikingly, E_2 stimulation of cardiomyocytes resulted in ANF expression in 64±8% of the cells ($P<0.05$ versus serum-free; Figure 2F).

The induction of ANF after E_2 stimulation was confirmed by Northern blot analyses. We showed an approximately 2-fold induction in ANF mRNA expression after either serum (10% FBS) or PE stimulation. E_2 stimulation alone of NRVMs resulted in a 5-fold induction in ANF mRNA, an effect that could be abrogated in the presence of the ER antagonist ICI 182,780.

To address the requirement of extracellular presence of ANF in the antagonistic effects of E_2 on cardiomyocyte hypertrophy, an antibody directed against ANF was added in increasing concentrations. The E_2 antagonism of cardiomyocyte hypertrophy was abrogated in a dose-dependent manner. In addition, PE-treated cardiomyocytes showed a dose-dependent increase in the overall surface area compared with PE treatment alone (Figure 3A). The presence of the antibody led to reduced bioavailability of ANF (Figure 3B).

To further dissect the role of E_2 in the transcriptional control of the ANF gene, ANF promoter deletion fragments (linked to a luciferase reporter) were tested for their ability to induce luciferase expression after E_2 stimulation. A deletion fragment of 700 bp of the ANF promoter demonstrated substantial transactivation after E_2 treatment, and this effect was attenuated in the presence of ICI 182,780. The proximal 150-bp fragment of the ANF promoter showed a reduced response to E_2, suggesting that the estrogen-responsive transactivation elements are located within the region between −150 and −700 bp of the proximal promoter (Figure 4).

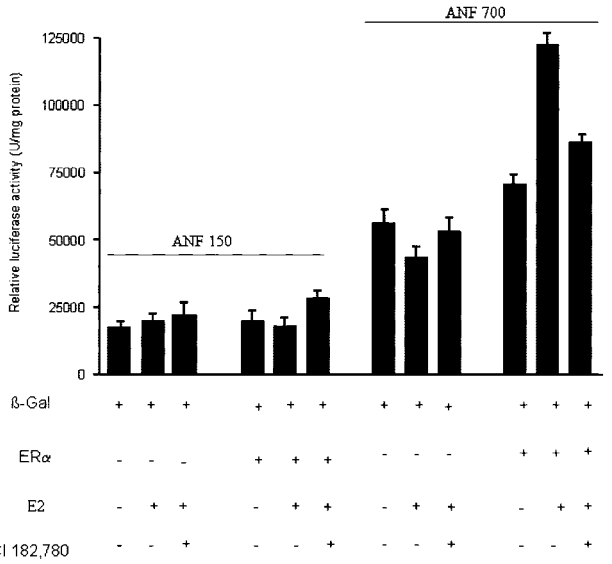

Figure 4. Results of transient transfection using ANF promoter deletion fragments in cultured HEK293 cells. ANF promoter fragments included −150 and −700 bp of proximal ANF promoter. After stimulation with 17β-estradiol (10^{-9} mol/L) and/or ICI 182,782 (10^{-5} mol/L) for 48 hours.

E_2 Activates the GC-A Receptor

Accumulation of ANF may result in autocrine/paracrine stimulation of NRVMs through its cognate transmembrane GC-A receptor. E_2 and ANF increased cGMP activity by 14.58±3.5 and 26.21±5.8 pmol/mg protein, respectively, compared with control (below detection level). We found that administration of either ANF (10^{-5} mol/L) or 8-Br-cGMP (10^{-5} mol/L) resulted in attenuation of cardiomyocyte hypertrophy. Both agents decreased cardiomyocyte surface area by 50% (Figure 5A, c and e) compared with PE stimulation alone (Figure 5A, b). The level of inhibition achieved by ANF or 8-Br-cGMP addition was comparable with that resulting from coadministration of E_2 (10^{-9} mol/L; Figure 5A, g). Quantification of the cell-surface areas supported the immunocytochemical observations (Figure 5B).

Cardiomyocytes were stimulated with PE and E_2, ANF, or 8-Br-cGMP in the presence of the cGMP-dependent protein kinase (cGK) blocker KT-5823 (10^{-6} mol/L). KT-5832 completely abolished the antagonistic growth potential of E_2, ANF, or 8-Br-cGMP of cardiomyocytes after PE administration (Figure 5A, d, f, and h). Indeed, PE stimulation in the presence of cGK inhibition resulted in significantly larger cardiomyocytes compared with those stimulated with PE alone (Figure 5A, b and i). This property of KT-5823 was not because of growth-evoking properties of KT-5823 alone (Figure 5A, j) or specific to PE stimulation alone, because potentiation of cardiomyocyte hypertrophy was also observed after stimulation with ET-1 (10^{-7} mol/L) or CT-1 (10^{-9} mol/L) in the presence of KT-5823 (data not shown). Quantification of the cell-surface areas supported the immunocytochemical observations (Figure 5B).

Discussion

Estrogen Antagonizes Cardiomyocyte Hypertrophy

Various observations suggest that estrogen may play an important role in modulating cardiac hypertrophy.[15] Gender-related differences have been observed in the development of pressure-overload hypertrophy.[16] Recently, we were able to demonstrate that estrogen attenuates the development of pressure-overload hypertrophy. An interesting correlation between ANF expression, hypertrophy, and estrogen was observed.[6] The present study provides, for the first time, evidence that estrogen modulates ANF through a genomic, ER-dependent pathway in NRVMs. Estrogen-induced ANF accumulation in the ventricular myocyte most likely results in ANF receptor activation in an autocrine/paracrine manner, which in turn evokes cytoplasmic cGMP signaling downstream of the GC-A receptor. Thus, E_2 increases the expression of ANF, which antagonizes LVH.

Estrogen and ANF Induction

ANF gene expression in ventricular myocytes occurs in response to diverse hypertrophic stimuli in multiple mammalian species, including humans. ANF induction has therefore

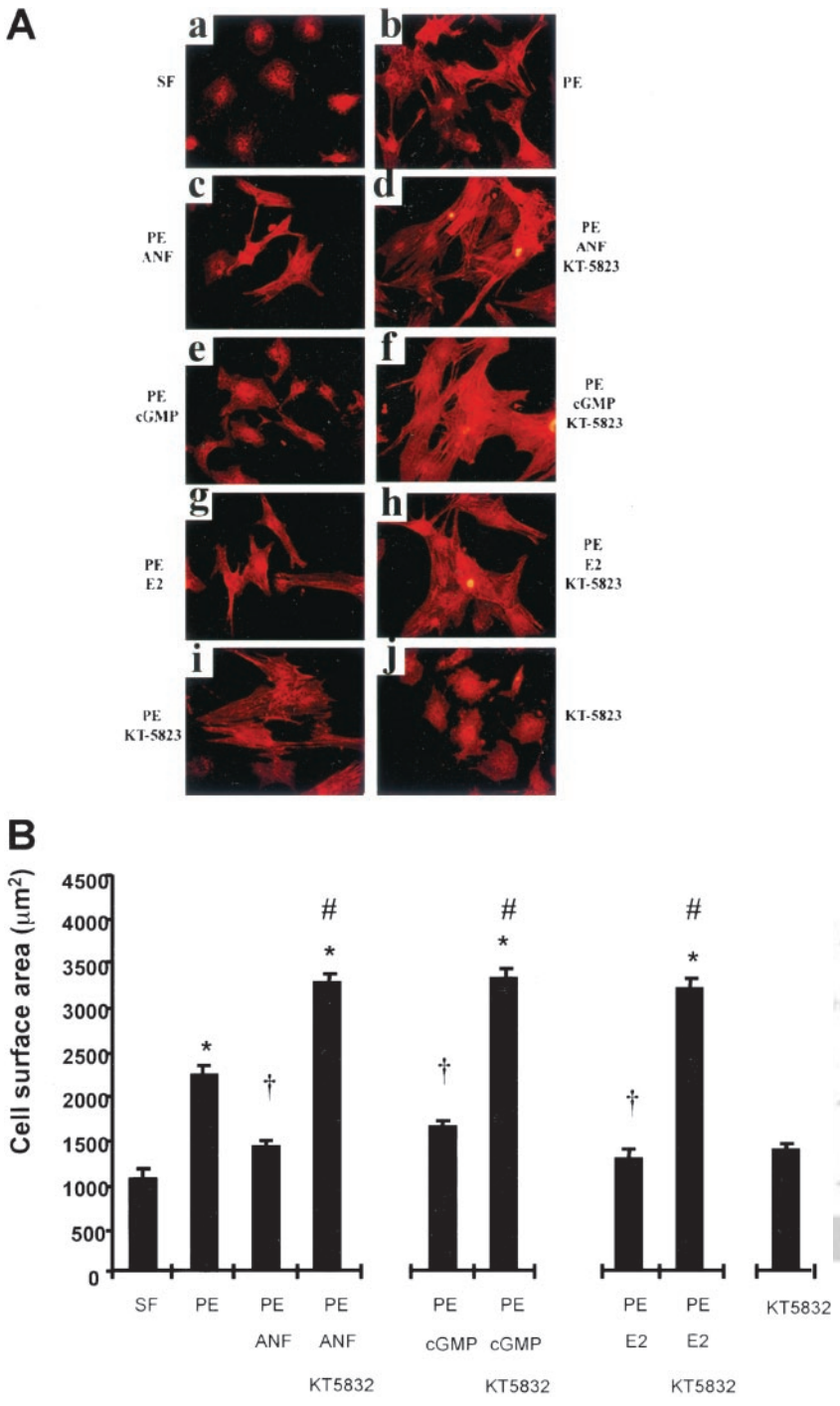

Figure 5. A, Antihypertrophic effects of estrogen involve autocrine stimulation of GC-A receptor and cGMP. Cardiomyocytes were identified with α-actinin antibody (red signal), and nuclei were stained with bisbenzamide (white). Cardiomyocytes stimulated with PE (10^{-5} mol/L) demonstrated a significant hypertrophic response (b) compared with myocytes cultured under control (−) conditions (a). Stimulation of GC-A receptor with ANF (10^{-5} mol/L) resulted in a significant attenuation of PE-induced cardiomyocyte hypertrophy (c). Similarly, addition of 8-Br-cGMP (10^{-5} mol/L) blocked PE-mediated cardiomyocyte hypertrophy (e). Effects of ANF and cGMP were comparable to that of E_2 (g). Antihypertrophic effects of ANF or 8-Br-cGMP could be reversed by inhibition of cGK with KT5823 (10^{-6} mol/L) (d, f, and h). Cardiomyocytes cultured in presence of PE and K-T5823 alone demonstrated a more massive hypertrophic response (i) than PE-stimulated cardiomyocytes (b). B, Quantification of cell surface area of 3 independent experiments. *$P<0.05$ vs serum-free (SF) conditions, †$P<0.05$ vs PE.

been considered to be one of the conserved molecular features of ventricular cell hypertrophy. In human failing and hypertrophied hearts, the expression of ANF is markedly induced, and considerable levels of the peptide are detected in cardiomyopathic ventricles.[17] The present findings demonstrate that estrogen is able to induce ANF gene expression. Because we were not able to find a consensus ER-binding element in the proximal ANF promoter, it is feasible that ERs may influence ANF promoter activity indirectly through interactions with other cofactors, such as Sp-1.[18] In this light, it is of interest that multiple important *cis*-acting elements, such as Sp1 sites, have been recognized in the ANF promoter.[19] In fact, transcriptionally active Sp1-ER complexes have been identified and shown to influence promoter activity of other genes, thereby providing a possible explanation of how ER may influence transactivation of the ANF promoter in the absence of a functional ER-binding element.[20] Previously, Hong et al[21] demonstrated that ovariectomy decreased atrial ANF mRNA transcripts in rats. Female Wistar rats treated with estrogen demonstrated increased ANF gene expression. The present study supports the notion that estrogen may be an important factor for transactivation of the ANF gene.

ANF and Cardiomyocyte Hypertrophy

Previous studies have already provided substantial evidence for an antagonistic role of ANF on the development of LVH. On several accounts, ANF was shown to possess growth-inhibitory properties on cultured NRVMs.[9] Calderone et al[22] demonstrated that exogenous ANF and cGMP inhibit the protein synthesis in NRVMs.

Single nucleotide polymorphisms within the first 650 bp of the ANF promoter gene of Wistar-Kyoto rats were demonstrated to influence promoter activity, in keeping with a higher LV ANF concentration under basal conditions compared with Wistar-Kyoto Hyperactive rats with a larger LV mass.[23] That study showed that rats with lower ANF levels had an increased LV mass. Genetically modified mice with complete absence of the GC-A receptor demonstrated elevated blood pressure and marked cardiac hypertrophy with interstitial fibrosis,[24] thereby illustrating the importance of the GC-A receptor for the suppression of LVH. Mice with a disrupted pro-ANF gene lack circulating and tissue ANF and exhibit increased heart weight and blood pressure when maintained on intermediate-salt diets,[25] again showing the importance of ANF signaling to block LVH. Conversely, hearts from mice with cardiac-restricted overexpression of the GC-A receptor were smaller than their wild-type counterparts and had distinct antihypertrophic properties independent of vascular tone.[26] Taken together, these studies support the relevance of the ANF GC-A receptor pathway to prevent hypertrophy.

cGMP-Dependent Protein Kinase and Cardiomyocyte Hypertrophy

New observations suggest that cGMP signaling may play a crucial role in the antihypertrophic effects of estrogen and/or ANF on NRVMs. Estrogen rapidly activates calcium-activated potassium channels. Such activation occurs through a pathway dependent on nitric oxide (NO) and cGMP.[27] Furthermore, most of the actions of ANF are mediated through activation of its transmembrane GC-A receptor.[28] Receptor-generated cGMP binds to cGK, which is thought to mediate the principal biological functions of cGMP. Estrogen also stimulates NO production through activation of endothelial NO synthase via nongenomic and genomic effects.[29] This may be associated with synergistic stimulation of cGK in cardiomyocytes. Both stimulations through ANF induction or endothelial NO synthase activation result in blocking hypertrophy.

Here we report that E_2 treatment counterbalanced the morphological and biochemical parameters of myocyte hypertrophy after stimulation with PE or ET-1. A marked upregulation of ANF mRNA and protein levels that accompanied the morphological observations mediated through an ER-mediated pathway. Treatment with E_2 activates the ANF receptor in an autocrine/paracrine fashion, which leads to increased activation of cGMP and cGK. In summary, we show here that estrogen is a critical mediator of ANF regulation in cardiac hypertrophy. These observations may help us to understand the gender-based differences found in cardiac disease.

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