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Role for Early Growth Response-1 Protein in α_1-Adrenergic Stimulation of Fibroblast Growth Factor-2 Promoter Activity in Cardiac Myocytes

YAN JIN, FARAH SHEIKH, KAREN A. DETILLIEUX, and PETER A. CATTINI

Gene Technology and Departments of Physiology, and Pharmacology and Therapeutics, University of Manitoba, Winnipeg, Manitoba, Canada

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ABSTRACT

Fibroblast growth factor-2 (FGF-2), a mitogenic, angiogenic, and cardioprotective agent, is released from the postnatal heart by a mechanism of transient remodelling of the sarcolemma during contraction. Both release of FGF-2 and its synthesis can be increased with adrenergic stimulation. We reported previously that FGF-2 synthesis can be regulated at the transcriptional level by α-adrenergic stimulation of cultured neonatal rat cardiac myocytes as well as in the adult mouse heart. Examination of the proximal promoter region of both human and rat FGF-2 gene sequences revealed binding sites for the early growth response-1 (Egr-1) protein. Using gel mobility shift assays, we observed a transient increase in a complex between nuclear extracts from neonatal rat cardiac myocytes treated with inducers of Egr-1, including the α-adrenergic agonist phenylephrine, angiotensin II, and phorbol ester, and a consensus Egr-1 DNA element. A similar complex was seen with the FGF-2 promoter region $-7/+42$ as the DNA probe, but not when the Egr-1 element at nucleotides $+3/+31$ was disrupted. Participation of Egr-1 protein in the complex was confirmed by competition with Egr-1 DNA elements and antibodies. With deletion analysis and transfection of neonatal rat cardiac myocytes, the α-adrenergic response was localized to nucleotides $-110/+42$ of the FGF-2 gene in the context of a hybrid FGF-2/luciferase reporter gene, -110FGFp.*luc*. Overexpression of Egr-1 increased -110FGFp.*luc* gene expression, whereas mutation of its Egr-1 element at nucleotides $+3/+31$ abolished α-adrenergic responsiveness. These data indicate that Egr-1 is involved in the α-adrenergic stimulation of the FGF-2 promoter region in neonatal cardiac myocytes.

The catecholamines norepinephrine (NE) and epinephrine play important roles in the control of normal cardiac function and arterial blood pressure (Terzic et al., 1993). Cardiac myocytes possess both α_1- and β-adrenoceptor subtypes. Under physiological conditions, the effect of catecholamines on the heart are mediated primarily by β-adrenoceptors (Will-Shahab and Shubert, 1991). However, β-adrenoceptor-mediated responses are impaired under certain pathological conditions such as myocardial infarction, congestive heart failure, dilated cardiomyopathy, and thyroid hypofunction (Will-Shahab and Shubert, 1991). This has led to the idea that "stimulation of the α-adrenoceptors might serve as a reserve mechanism and/or might have a compensatory role in maintaining myocardial responsiveness to catecholamines under pathological conditions" (Will-Shahab and Shubert, 1991). There is evidence that ischemic preconditioning in the isolated rat heart is mediated by α_1-adrenoceptors (Banerjee et al., 1993). Thus, targeting genes that can be induced by α-adrenergic stimulation offers a potential approach to limit cardiac injury.

We reported previously that basic fibroblast growth factor (FGF-2) synthesis can be increased at the transcriptional level in the heart after α_1-adrenergic stimulation via NE or the α_1-specific agonist phenylephrine (Detillieux et al., 1999). FGF-2 acts as a regulator of myocardial and vascular cell growth and differentiation, both normally and in response to injury (Scheinowitz et al., 1997). FGF-2 mediates various biological responses, including mitogenesis and angiogenesis (Bikfalvi et al., 1997), through the binding of specific cell surface high-affinity tyrosine kinase receptors (fibroblast growth factor receptor-1) and low-affinity sites (Klagsbrun and Baird, 1991; Jaye et al., 1992). FGF-2 and fibroblast growth factor receptor-1 are essential for normal cardiac development (Mima et al., 1995; Leconte et al., 1998) and are present in the heart into adulthood (Jin et al., 1994; Liu et

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ABBREVIATIONS: NE, norepinephrine; FGF-2, fibroblast growth factor-2; Egr-1, early growth response-1; FBS, fetal bovine serum; CMV, cytomegalovirus; SV40, simian virus 40; EMSA, electrophoretic gel mobility shift assay; PMA, phorbol-12-myristate-13-acetate; ATII, angiotensin II; WT, wild type; MUT, mutation; PE, phenylephrine; DMEM, Dulbecco's modified Eagle's medium; Praz, prazosin.

al., 1995). The role of FGF-2 in the postnatal heart is unclear, however, both increased and decreased FGF-2 levels have been used to prevent or reduce damage to the cardiovascular system through effects on different cell types at different stages of the stress/injury process. Increased FGF-2 levels, through exogenous addition, were shown to protect cardiac myocytes from injury by free radicals (Kardami et al., 1993) as well as act in a cardioprotective manner in models of heart ischemia/reperfusion in the rat (Padua et al., 1995; Cuevas et al., 1997; Padua et al., 1998). FGF-2 treatment also improved myocardial function in ischemic porcine, canine, and human hearts through angiogenesis/increased collateral blood flow and a reduction in the size of the infarct (Yanagisawa-Miwa et al., 1992; Harada et al., 1994; Laham et al., 1999). Conversely, decreased FGF-2 levels, through the use of antisense RNA, induced apoptosis in vascular smooth muscle cell cultures (Fox and Shanely, 1996), and reduced neointimal thickening after balloon catheter carotid artery injury (Hanna et al., 1997). Thus, regulation of endogenous FGF-2 levels in the heart, as seen with adrenergic stimulation, might contribute to the maintenance of a healthy cardiovascular system as well as limit the extent of damage and improve recovery from an ischemic episode.

The basic regulatory mechanism underlying α_1-adrenergic stimulation of FGF-2 synthesis is not known. A comparison of human (Biesiada et al., 1996; Wang et al., 1997) and rat (Pasumarthi et al., 1997) FGF-2 gene sequences revealed binding sites for early growth response-1 (Egr-1) protein in the proximal promoter region. Egr-1, also known as NGFI-A, krox24, and TIS8, is a member of the (three) zinc finger family of transcription factors (Biesiada et al., 1996). It is an example of a product of primary response or intermediate-early genes that are induced by mitogenic stimuli, such as serum and phorbol esters, and often constitutes the first step in the sequential expression of growth regulatory proteins (Biesiada et al., 1996). The induction of Egr-1 synthesis on α_1-adrenergic stimulation of neonatal rat cardiac myocytes by NE treatment has been reported (Iwaki et al., 1990). Although available data suggest a major regulatory role for this transcription factor and FGF-2 (Biesiada et al., 1996), there has been no report of Egr-1-mediated control of the FGF-2 promoter in cardiac cells.

We have investigated a possible role for Egr-1 in the α_1-adrenergic stimulation of the FGF-2 promoter in neonatal cardiac myocytes. We show that 1) Egr-1 binding to the FGF-2 promoter region is induced after α-adrenergic stimulation, 2) an increase in Egr-1 levels stimulates FGF-2 promoter activity, and 3) mutation of the Egr-1 site blocks α-adrenergic stimulation of the FGF-2 promoter region. These results are discussed in terms of a role for adrenergic stimulation of FGF-2 in the normal maintenance of the heart and its response to injury.

Materials and Methods

Cell Culture. Neonatal rat cardiac ventricular myocyte cultures were prepared according to established procedures (Iwaki et al., 1990; Detillieux et al., 1999). Ventricles were dissected from rat pups at 1 to 36 h after birth and the cells enzymatically disaggregated in a spinner flask with trypsin and DNase I. Myocytes were fractionated from nonmuscle cells on a discontinuous Percoll gradient and plated on collagen-coated plates at a density of 1.2×10^6 cells/60-mm dish. Cells were initially plated in Ham's F10 medium containing 10% fetal bovine serum (FBS), 10% horse serum, and antibiotic (1000 U/ml penicillin and 1 mg/ml streptomycin).

Plasmids and Constructs. The hybrid gene −1058FGFp.*luc*, containing the region −1058/+54 of the rat FGF-2 gene fused upstream of a promoterless firefly luciferase gene in the vector pXP1, was described previously (Pasumarthi et al., 1997). The fragment corresponding to −911/+42 of the rat FGF-2 gene was generated by polymerase chain reaction with10 ng of template (−1058FGFp.*luc* gene) and primers P1, 5′-GGGCTGGAGGGCGGTGAGCACAGATCTTTAATCACAGCGCTGGAGGC-3′ and P2, 5′-ACCCCGCGTCCCCGCCTGCACCCCGGCCCCGCGCGCCCCCGCCCCGCGC-3′. The product was digested with *Bgl*II to produce a fragment with *Bgl*II (underlined) and blunt ends, and inserted into pXP1 (Nordeen, 1988) cut with *Bgl*II/*Sma*I to generate −911FGFp.*luc*. To generate −110FGFp.*luc*, −911FGFp.*luc* was digested with *Dra*I and *Hin*dIII to release a fragment containing −110/+42, which was resolved by 4% agarose gel electrophoresis, isolated, and inserted into pXP1 cut with *Sma*I/*Hin*dIII. Production of the hybrid −110FGF/MUTp.*luc* gene was identical with −110FGFp.*luc*, except in the initial polymerase chain reaction stage, primer P1 was paired with P3, 5′-ACCCCGCGTCCTAGCCTGCACCCCGGCCTAGCGCGCCCTAGCCCCGCGC-3′, which contains mutated sequences (underlined). Hybrid genes containing the firefly luciferase gene directed by the cytomegalovirus promoter (CMVp.*luc*) or the chloramphenicol acetyl transferase gene directed by the simian virus 40 promoter (SVp.*cat*) are described elsewhere (Lytras and Cattini, 1994). The Egr-1 expression vector (CMVp.Egr-1) was a generous gift from Dr. Vikas P. Sukhatme (Beth Israel Hospital, Boston, MA). The "empty" expression vector pcDNA3, containing the CMV promoter, is commercially available (Invitrogen, San Diego, CA).

Electrophoretic Gel Mobility Shift Assay (EMSA). The nuclear protein was prepared from cultured neonatal rat cardiac myocytes treated without or with 0.1 μM phorbol-12-myristate-13-acetate (PMA), 0.1 μM angiotensin II (ATII), and 0.1 mM phenylephrine (PE) for 1 to 24 h (as indicated in the text or figures) as previously described (Andrews and Faller, 1991; Detillieux et al., 1998). The EMSA was done with an established protocol (Lytras and Cattini, 1994; Detillieux et al., 1998). The myocyte nuclear protein (2 μg) was incubated with 1 μg of poly(dI-dC) and ^{32}P-labeled DNA fragments (1 ng; 1×10^4 cpm). The sequences of one strand from each of the DNA fragments [consensus Egr-1 element, FGF-2-wild type (WT), and FGF-2-mutation (MUT)] used as probes are shown in Fig. 1. Reactions were done in binding buffer (10 mM HEPES-KOH pH 7.9; 210 mM NaCl; 6.5 mM MgCl; 1 μM $ZnSO_4$; 0.1 mM EDTA; 12.5% glycerol; 1 mM dithiothreitol; and 1 mM phenylmethylsulfonyl fluoride)

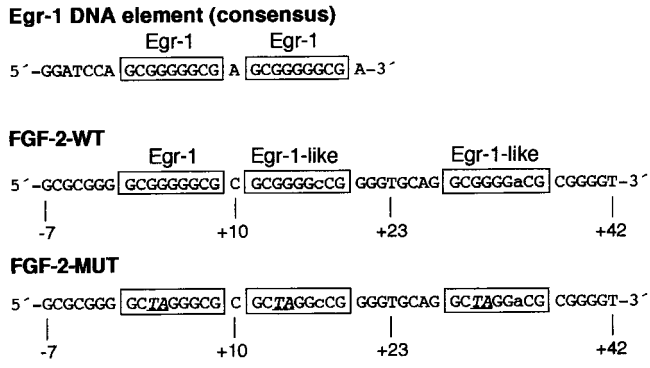

Fig. 1. Fragments containing Egr-1 or Egr-1-like sequences used as DNA probes for EMSA. The sequences of one strand of the region −7/+42 of the rat FGF-2 gene without (FGF-2-WT) or with (FGF-2-MUT) mutations of Egr-1 or Egr-1-like elements, as well as a commercially available consensus Egr-1 DNA element are shown. The Egr-1-related elements are boxed, and nucleotides that are not identical with the consensus Egr-1 site are shown as lowercase characters. The position of mutated nucleotides in FGF-2-MUT are indicated by both italics and underlining.

for 30 min at room temperature (20-μl final volume). For competition with DNA elements, competitor double-stranded oligonucleotides corresponding to consensus Egr-1 (Fig. 1) or Sp1 (5′-ATTC-GATCGGGGCGGGGCGAGC-3′) DNA elements (Santa Cruz Biotechnology, Santa Cruz, CA) were added with nuclear extract for 10 min at room temperature and then radiolabeled probe for an additional 20 min. For competition with specific antibodies, Egr-1 (1 μg) and Sp1 (2 μg) rabbit polyclonal IgGs (Santa Cruz Biotechnology), or the equivalent amount of normal rabbit serum was added to the binding reaction (30-μl final volume) after 20-min preincubation of the other components at room temperature, and incubated for an additional 10 min. The DNA-protein complexes were resolved in nondenaturing 5% polyacrylamide gels. All assays were done at least two times with two independently generated series of nuclear extracts.

Transient Gene Transfer. Neonatal rat cardiac myocytes were transfected by the calcium phosphate-DNA precipitation method as previously described (Detillieux et al., 1999). Briefly, 60 μg of plasmid was made up to a volume of 1.5 ml in 252 mM $CaCl_2$ and added gradually to an equal volume of aerated 2× HEBS buffer (280 mM NaCl; 50 mM HEPES-KOH, pH 7.10; and 1.5 mM Na_2PO_4). For cotransfection experiments to assess the effect of Egr-1 overexpression, the final volume was 3.0 ml, and 60 μg of reporter gene and 30 μg of CMVp.Egr-1 or pcDNA3 were used. Precipitate was allowed to form at room temperature for 30 min, and 500 μl was added to each of six culture dishes (60 mm) containing 5 ml of Dulbecco's modified Eagle's medium (DMEM)/10% FBS. Following a 16-h transfection period, the cells were washed thoroughly with calcium- and magnesium-free PBS, and refed with DMEM-F12 containing 10% FBS and antibiotics, and harvested 24 h later. For assessing α-adrenergic stimulation, the medium was changed at 16 h to DMEM-F12 that contained 1× insulin-transferrin-selenrum (Life Technologies, Gaithersburg, MD), 0.2% Albu Maxi (Life Technologies, Paisley, Scotland), 0.02 mg/ml ascorbic acid, and antibiotics. These "identical" plates of transfected myocytes were then treated without or with 0.01 mM NE in the presence or absence of the α-antagonist (0.01 mM) prazosin (Praz) for 6 h to ensure a direct comparison of promoter activity in untreated and treated cells. Cotransfection with a vector to assess DNA uptake was not done because several promoters used frequently for this purpose, including Rous sarcoma, CMV, and thymidine kinase were found to respond to α-adrenergic stimulation (Detillieux et al., 1998, 1999). The use of "identical" plates that have received the same calcium phosphate-DNA precipitate allows a specific construct to be assessed for responsiveness to a particular treatment such as NE, but does not permit the comparison of activities between constructs. Thus, results are expressed as fold response of a promoter construct to NE treatment relative to control levels (NE + Praz).

Reporter Gene Assays. After stimulation, transfected cardiac myocytes were harvested in calcium- and magnesium-free-PBS with a rubber policeman, pelleted, and lysed in 100 mM Tris-HCl, pH 7.8, containing 0.1%Triton X-100. Insoluble material was removed by centrifugation and the luciferase activity in the supernatant was measured using the Promega luciferase assay system (Fisher Scientific, Ontario, Canada) and a luminometer according to the manufacturer's instructions. Luciferase activity was normalized against lysate protein content as determined by the Bradford assay (Bio-Rad Laboratories, Ontario, Canada) to give values of luciferase activity per microgram of protein. Chloramphenicol acetyl transferase activity (counts per minute per microgram of cell lysate protein) for cells transfected with SVp.*cat* was measured as previously described (Lytras and Cattini, 1994). With values from at least three independent transfections, means and standard errors of the means (used to determine error bars) were assessed for each experiment. Finally, the value for each construct was expressed as fold effect relative to its own control that was arbitrarily set to 1.0.

Statistical Analysis. Data presented in the text and figures are mean values ± S.E. Statistical analysis of the results was carried out using the Student t (parametric) or Mann-Whitney (nonparametric) tests. In all cases, a value was considered statistically significant if P was determined to be $\leq .05$.

Results

Nuclear Protein Binding to an Egr-1 DNA Element Is Transiently Increased in Response to PE, ATII, and PMA Treatment of Cardiac Myocytes. As previously reported, both the human and rat FGF-2 genes contain consensus Egr-1 DNA elements in their upstream regions flanking coding sequences (Biesiada et al., 1996; Pasumarthi et al., 1997; Wang et al., 1997). The sequence and the relative location of these putative functional elements in the rat FGF-2 gene are shown in Fig. 1. It has been reported that PMA (Cheng et al., 1994), ATII (Neyses et al., 1993), and the α-adrenergic agonist PE (Iwaki et al., 1990) increase Egr-1 mRNA levels. In the latter case, the positive effect on Egr-1 synthesis was observed in rat neonatal cardiac myocytes. We examined whether this stimulation of Egr-1 RNA and protein levels might translate into an increase in levels of cardiac myocyte nuclear protein binding to a consensus Egr-1 DNA element. Neonatal rat cardiac myocytes were isolated and treated without or with 0.1 mM PE for 1 and 8 h; 0.1 μM ATII for 1, 2, and 8 h; and 0.1 μM PMA for 1, 2, 4, and 8 h. Nuclear extracts were generated from each of these treatment groups and then used in combination with a consensus Egr-1 DNA element (Fig. 1) as a probe in EMSAs (Fig. 2A). In each case, several complexes were observed with different mobilities. However, one of the low-mobility complexes, which was evident within 1 to 2 h of treatment with all three of the reagents used, declined and was barely detectable after 8 h (see arrowhead in Fig. 2A).

This low-mobility complex was most easily seen with extracts from cardiac myocytes treated with PMA. Thus, extracts from neonatal rat cardiac myocytes treated without or with PMA for 1, 2, 4, 8, 12, and 24 h were used to assess binding of cardiac myocyte nuclear protein to the rat FGF-2 gene sequences (nucleotides −7/+42) containing the putative Egr-1 element (Fig. 1). Again, a low-mobility complex appeared transiently, peaking at 2 h before declining (see arrowhead in Fig. 2B).

Levels of Egr-1 Protein Binding to Rat FGF-2 Gene Sequences Increase in Response to Phorbol Ester and α-Adrenergic Stimulation of Neonatal Rat Cardiac Myocytes. An attempt was made to more directly assess whether the nuclear protein responsible for the transient increase in DNA binding on the FGF-2 sequences was Egr-1. To this end, nuclear extracts were prepared from neonatal rat cardiac myocytes treated with either PMA or PE for 2 h; a time point consistent with high levels of the protein-DNA complex. First, these extracts were used in an EMSA to compare the patterns generated with the rat FGF-2 fragment corresponding to nucleotides −7/+42 (FGF-2-WT) versus the same fragment in which the putative Egr-1 site (as well as two additional Egr-1-like sites) was disrupted by site-directed mutagenesis (FGF-2-MUT; Fig. 1). In contrast to the WT FGF-2 DNA, the "transient" low-mobility complex, as indicated by arrowheads, was not seen on the mutant FGF-2 DNA with nuclear protein from cardiac myocytes treated with PMA (compare lanes b and c, Fig. 3A) or PE (compare lanes b and c, Fig. 3B). Second, the transient complex was

competed efficiently by a 100-fold picomole excess of Egr-1 but not an unrelated Sp1 consensus DNA element (compare lane b with lanes d and e, Fig. 3, A and B). Finally, a specific antibody raised to 14 amino acid in the carboxy terminus of Egr-1 (Lehmann et al., 1999) and non cross-reactive with Egr-2, Egr-3, or Wilm's tumor protein, was used to confirm the presence of Egr-1 in the transient complex. Binding of a specific antibody to a protein in a complex can result in the presence of a "supershift" if its binding does not interfere with the interaction between the protein and radiolabeled DNA. As a consequence, the band in the gel corresponding to the original complex is decreased or lost. Alternatively, antibody binding might interfere with the ability of the protein to interact with DNA, effectively blocking or neutralizing formation of the original complex. The transient complex seen with the WT FGF-2 DNA was competed efficiently with a specific Egr-1 antibody (compare lane b with lane f, Fig. 3, A and B) but not an unrelated Sp1 antibody and/or normal rabbit serum. Thus, Egr-1 can complex with FGF-2 sequences.

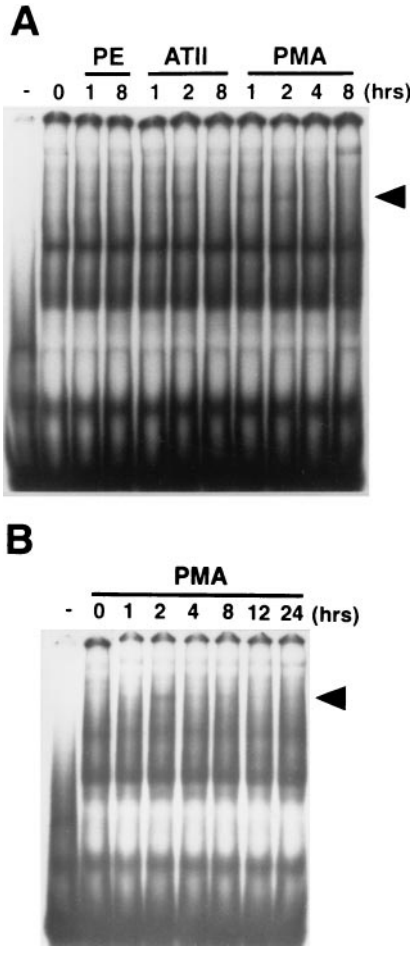

Fig. 2. Nuclear protein binding to DNA containing Egr-1 DNA elements is increased transiently in response to PE, ATII, and PMA treatment of neonatal rat cardiomyocytes. Isolated neonatal rat cardiac myocytes were treated without or with 0.1 mM PE for 1 and 8 h; 0.1 μM ATII for 1, 2, and 8 h; and 0.1 μM PMA for 1, 2, and 8 h as indicated (A). Nuclear extracts were generated from each of these treatment groups and then used in combination with a radiolabeled consensus Egr-1 DNA element (Fig. 1) as a probe in an EMSA. Although multiple complexes are evident after gel electrophoresis and autoradiography, one low-mobility complex (arrowhead) is increased transiently with PE, ATII, or PMA treatment. Extracts from neonatal rat cardiac myocytes treated without or with PMA for 1, 2, 4, 8, 12, and 24 h were used to assess binding of nuclear protein to the rat FGF-2 gene sequences (nucleotides −7/+42) containing the putative Egr-1 element, which was radiolabeled and used as a probe in an EMSA (B). After gel electrophoresis and autoradiography, the levels of a low-mobility complex (arrowhead) increase, peaking at 2 h before decreasing over the period of study. (A copy of the original figure is available on request).

Overexpression of Egr-1 Increases FGF-2 Promoter Activity in Transfected Rat Neonatal Cardiac Myocytes. We next assessed whether the activity of the FGF-2 promoter region could be stimulated via increased levels of Egr-1. The effect of Egr-1 overexpression on −1058FGFp.*luc,* −911FGFp.*luc*, and −110FGFp.*luc* expression in transfected neonatal rat cardiac myocytes was investigated. Hybrid FGF-2/luciferase and "control" reporter genes were cotransfected with expression vectors for Egr-1 (CMVp.Egr-1) or a vector (pcDNA3) containing the CMV promoter alone. The results are expressed in Fig. 4 as the fold effect of Egr-1 overexpression (+Egr-1) on the activity of each promoter relative to the value obtained after cotransfection with control pcDNA3 vector (−Egr-1). Significant 3.9 ($n = 7$; $P < .005$), 4.0 ($n = 6$; $P < .005$), and 5.4 ($n = 6$; $P < .05$) fold increases were observed in −1058FGFp.*luc*, −911FGFp.*luc*, and −110FGFp.*luc* gene expression, respectively. Although Egr-1 elicited a modest increase and decrease in SV40 and CMV promoter activities, respectively, these effects were not significant (Fig. 4).

***α*-Adrenergic Responsiveness Can Be Blunted after Disruption of Six Nucleotides in a 29-Base Pair Region (Nucleotides +3/+31) of Rat FGF-2 Gene.** Previously, we demonstrated that hybrid genes containing the regions −1058/+54, −911/+54, and −313/+54 of the rat FGF-2 gene were responsive to α-adrenergic stimulation of neonatal rat cardiac myocytes (Detillieux et al., 1999). To further localize this response, a more truncated region (−110/+42) in the context of the −110FGFp.*luc* gene was assessed. Cardiac myocytes were transfected with the −110FGFp.*luc* gene or, as a positive control, the −911FGFp.*luc* gene, and treated

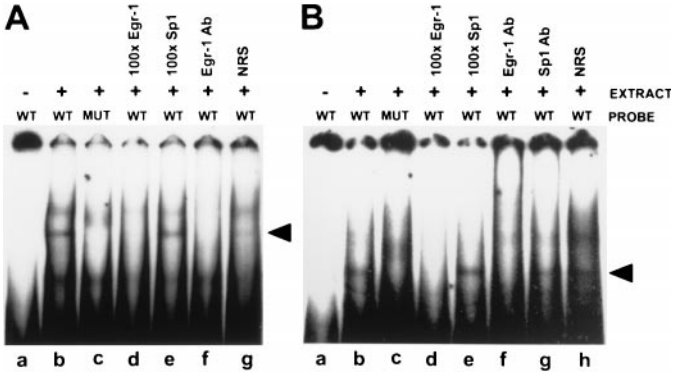

Fig. 3. Egr-1 binding to rat FGF-2 gene sequences is increased in response to PMA and PE treatment of neonatal rat cardiac myocytes. WT rat FGF-2 fragment corresponding to nucleotides −7/+42, or the same fragment with mutated Egr-1 sequences (MUT) were used as radiolabeled probes without (−) or with (+) nuclear extract from neonatal rat cardiac myocytes treated with either PMA (A) or PE (B) for 2 h, in EMSAs. After gel electrophoresis and autoradiography, the transient low-mobility complex (arrowhead) can be seen in both treatment groups with the WT probe (lane b), but not the MUT probe (lane c). For competition, the transient complex was competed efficiently by a 100-fold picomole excess of Egr-1 (lane d) but not an unrelated Sp1 consensus DNA element (lane e). The transient complex also competed efficiently with an Egr-1 antibody (lane f) but not an unrelated Sp1 antibody or normal rabbit serum (lanes g and h).

with the natural catecholamine (0.01 mM) NE versus NE and the α-specific antagonist (0.01 mM) Praz for 6 h. The results are expressed in Fig. 5 as the fold α-adrenergic effect of NE (+NE) on the activity of each promoter relative to the value obtained in the presence of NE and Praz (NE + Praz). NE treatment resulted in comparable and significant 2.7- and 3.2-fold increases in −110FGFp.*luc* ($n = 6$; $P < .005$) and −911FGFp.*luc* ($n = 7$; $P < .001$) gene expression, respectively. These effects appear to be α-specific because there was no significant difference between the values obtained with NE in the presence of Praz (NE + Praz) and without NE (−NE) treatment, that also was assessed for comparison.

We then assessed the importance of the Egr-1 DNA element (Fig. 1) and binding activity (Fig. 3) in the region −7/+42 of the FGF-2 gene. Sequences at nucleotides +3/+31 of the −110FGFp.*luc* gene were disrupted by site-directed mutagenesis (Fig. 1), to generate the −110FGF/MUTp.*luc* gene. This mutant gene was then tested for α-adrenergic responsiveness as described for the above-mentioned WT gene. Again, the results are expressed in Fig. 5 as the fold α-adrenergic effect of NE (+NE) on the activity of the −110FGF/MUT promoter relative to the value obtained in the presence of NE and Praz (NE + Praz). The mutation of the Egr-1 DNA element resulted in the loss of significant α-adrenergic responsiveness with NE treatment.

Discussion

We reported previously that FGF-2 synthesis can be regulated at the transcriptional level by α-adrenergic stimulation of cultured neonatal rat cardiac myocytes (Detillieux et al., 1999). This was done using NE in combination with the α-specific antagonist Praz, as well as PE. The presence of this pathway in vivo was suggested by experiments with transgenic mice containing the rat FGF-2 promoter region fused to the firefly luciferase reporter gene (Detillieux et al., 1999). This study was restricted to an assessment of the effect of α-adrenergic stimulation on transgene expression in "whole" heart. Subsequently, we have been able to confirm that rat FGF-2 promoter activity can be stimulated by NE and phorbol ester treatments in isolated and cultured adult cardiac myocytes from −1058FGFp.*luc* transgenic mice (F.S. and P.A.C., unpublished data).

Our initial attempt to localize the sequences responsible for the α-adrenergic response focused on A/G-rich sequences that were implicated in the adrenergic responsiveness of the atrial natriuretic factor promoter (Ardati and Nemer, 1993). A pair of these sequences is located 780 and 761 base pairs upstream of a major transcription initiation site in the rat FGF-2 promoter region. These were ruled out by heterologous promoter (Detillieux et al., 1998) and DNA deletion studies (Detillieux et al., 1999), which suggested that sequences contained, at least in part, between nucleotide position −313 and +54 of the rat FGF-2 promoter region were involved in the response to NE or PE (Detillieux et al., 1998, 1999).

Like its human homolog, the rat FGF-2 gene promoter does not contain typical TATA or CAAT boxes (Shibata et al., 1991; Pasumarthi et al., 1997). Instead, activation of transcription may involve binding of factors to a GC-rich region that has been associated with the presence of transcription initiation sites (Biesiada et al., 1996; Pasumarthi et al., 1997). GC-rich regions in both human (Biesiada et al., 1996; Wang et al., 1997) and rat (Pasumarthi et al., 1997) FGF-2 proximal promoter regions contain Egr-1-binding sites. In the rat, Egr-1 and Egr-1-like DNA elements are located between nucleotides +1 and +36 of the FGF-2 promoter region (Fig. 1). Our data indicate that α-adrenergic stimulation of

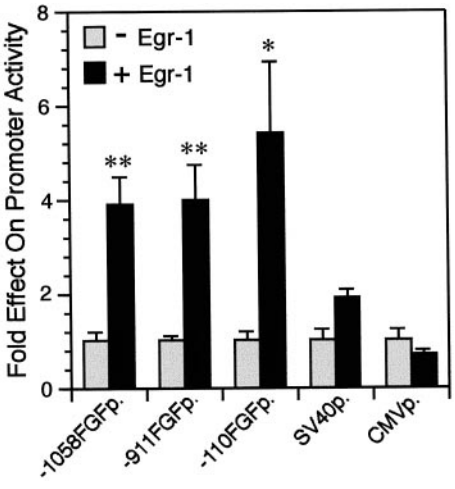

Fig. 4. Overexpression of Egr-1 increases FGF-2 promoter region activity in neonatal cardiac myocytes. Neonatal rat cardiac myocytes were transfected with hybrid luciferase genes directed by truncated regions of the rat FGF-2 promoter region (−1058/+54, −911/+42, and −110/+42), and cotransfected with (+Egr-1) or without (−Egr-1) an Egr-1 expression vector. Hybrid reporter genes directed by the SV40 or CMV promoter (p.) also were assessed. The results are presented as the mean fold effect on promoter activity in the presence of Egr-1 overexpression (+Egr-1/−Egr-1), and the values for −Egr-1 have been arbitrarily set to 1.0 ($n = 3$–7). The error bars indicate the standard error from the mean. For level of significance, $^{**}P < .01$ and $^{*}P < .05$.

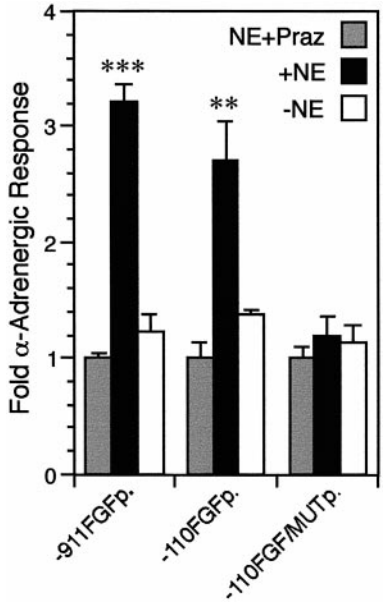

Fig. 5. α-Adrenergic responsiveness is lost after disruption of the Egr-1 element in the FGF-2 promoter region. Neonatal rat cardiac myocytes were transfected with the −911FGFp.*luc*, −110FGFp.*luc*, or −110FGF/MUTp.*luc* gene, and treated without (−NE) or with NE in the absence (+NE) or presence of the α-antagonist Praz (NE + Praz). The results (luciferase activity per microgram of protein) are expressed as the mean fold response of each promoter to α-adrenergic stimulation (NE/NE + Praz), and the values for NE + Praz have been arbitrarily set to 1.0 ($n = 5$–7). The error bars indicate the standard error from the mean. For level of significance, $^{***}P < .001$ and $^{**}P < .01$.

postnatal cardiac myocytes results in an increase in FGF-2 promoter region activity via Egr-1 and specifically, a direct effect on Egr-1-responsive DNA sequences located in the 36-base pair region between nucleotide positions +1 and +36 (Fig. 1). This is supported largely by EMSA data demonstrating Egr-1 binding to this region (Fig. 3), the stimulation of the FGF-2 promoter region (−110/+42) with increased Egr-1 levels (Fig. 4), and the loss of α-adrenergic-responsiveness when these sequences (nucleotides +3/+4, +13/+14, and +30/+31) were mutated (Fig. 5).

Our results also suggest that effects exerted via Egr-1 would not be limited to α-adrenergic stimulation but might include additional stimuli of protein kinase C activity, including PMA and ATII (Fig. 2). For the platelet-derived growth factor-A promoter, a pathway from protein kinase C activation to Egr-1 induction (by PMA) has been proposed. On stimulation, extracellular regulated kinase and/or c-Jun-terminal kinase are activated, and in turn modify the ternary complex binding to the serum-responsive element located in the promoter of the Egr-1 gene (Khachigian and Collins, 1997). This results in an increase in Egr-1 expression and levels, stimulating platelet-derived growth factor-A promoter and gene expression (Khachigian et al., 1995; Khachigian and Collins, 1997). Interestingly, this final event involves displacement of a second transcription factor (Khachigian et al., 1995).

We cannot, of course, rule out the possibility of additional pathways for α-adrenergic stimulation of the FGF-2 promoter. This might involve an indirect effect of Egr-1 on other transcription factors, or even an Egr-1-independent mechanism, possibly involving sequences outside those assessed in this study. However, in this context, the −1058FGFp.*luc* transgene and endogenous mouse FGF-2 gene responded in a similar manner to α-adrenergic stimulation in the transgenic mouse heart (Detillieux et al., 1999). Also, the fold stimulation of −110FGFp.*luc* activity in response to increased Egr-1 levels or α-adrenergic stimulation was comparable to that seen with −1058FGFp.*luc* and/or −911FGFp.*luc* genes (Figs. 4 and 5). More importantly, because α-adrenergic stimulation of cardiac myocytes has already been shown to increase Egr-1 synthesis (Iwaki et al., 1990) and, in this study, bioavailability (Figs. 2 and 3), a direct effect of Egr-1 on FGF-2 promoter sequences offers the most likely mechanism to explain α-adrenergic regulation of FGF-2 gene expression in postnatal cardiac myocytes.

Prolonged α_1-adrenergic stimulation of neonatal rat cardiac myocytes cultures is known to induce hypertrophic growth and gene transcription (Rokosh et al., 1996 and references therein). Interestingly, FGF-2 has been implicated in induction of cardiac hypertrophy and is known to alter the pattern of contractile protein gene expression from an adult to a fetal program, a characteristic feature of pressure overload-induced hypertrophy (Rokosh et al., 1996). Thus, our finding that α_1-adrenergic stimulation increases FGF-2 synthesis (Detillieux et al., 1999) through, at least in part, transactivation by Egr-1, suggests a role for FGF-2 and this pathway in the hypertrophic response seen in neonatal rat cardiac myocyte cultures. The role for FGF-2-mediated hypertrophy in vivo is more controversial. Although mice with a targeted disruption of the FGF-2 gene were associated with a lesser degree of hypertrophy during pressure overload, chronic administration of FGF-2 in rats with myocardial infarction did not correlate with ventricular hypertrophy (Schultz et al., 1999).

The mitogenic, angiogenic, and cardioprotective properties of FGF-2 also make it a desirable target for activation in terms of offering maintenance as well as cardioprotective effects via adrenergic stimulation. FGF-2 is released from cardiac myocytes during contractions under normal physiological conditions as well as in paced cardiac myocytes in vitro (Clarke et al., 1995; Kaye et al., 1996). This occurs through contraction-induced transient remodeling of the myocyte plasma membrane (Clarke et al., 1995; Kaye et al., 1996). α_1-Adrenergic stimulation would be expected to increase contractility (Terzic et al., 1993) and, as a consequence, release of FGF-2. Thus, this could provide a mechanism by which FGF-2 is released from intracellular pools at the site of injury where it would contribute to protection of the myocardium. The question raised in our current study, concerned the basic regulatory mechanism underlying the increase in FGF-2 synthesis by α_1-adrenergic stimulation at the transcriptional level. Our results strongly implicate Egr-1 in the α_1-adrenergic responsiveness of the rat FGF-2 gene in cardiac myocytes. This might represent a necessary component for maintenance, the cardioprotective process, or a response to injury, through either replenishing intracellular stores or generating FGF-2 for immediate release.

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Send reprint requests to: Peter A. Cattini, Department of Physiology, University of Manitoba, 730 William Ave., Winnipeg, Manitoba, Canada R3E 3J7. E-mail: Peter_Cattini@UManitoba.CA