α1-Adrenergic stimulation of FGF-2 promoter in cardiac myocytes and in adult transgenic mouse hearts

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Detillieux, Karen A., Johanna T. A. Meij, Elissavet Kardami, and Peter A. Cattini. α1-Adrenergic stimulation of FGF-2 promoter in cardiac myocytes and in adult transgenic mouse hearts. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H826–H833, 1999.—Fibroblast growth factor (FGF-2), a mitogenic, angiogenic, and cardioprotective agent, is reported to be released from the postnatal heart by a mechanism of transient remodeling of the sarcolemma during contraction. This release can be increased with adrenergic stimulation. RNA blotting was used to assess whether FGF-2 synthesis in neonatal rat cardiomyocytes might also be regulated by adrenergic stimulation. FGF-2 RNA levels were increased after treatment with norepinephrine for 6 h or with the α1-adrenergic agonist phenylephrine for 48 h. To assess an effect on transcription, neonatal rat cardiomyocytes were transfected with a hybrid rat FGF-2 promoter/luciferase gene (−1058GF2p.luc) and treated with norepinephrine or phenylephrine for 6 or 48 h, respectively. FGF-2 promoter activity was increased two- to sevenfold in an α2-specific manner. Putative phenylephrine-responsive elements (PEREs) were identified at positions −780 and −761 relative to a major transcription initiation site. However, deletion analysis of −1058GF2p.luc showed that the phenylephrine response was independent of the putative PEREs, cell contraction, and Ca2+ influx. In transgenic mice expressing −1058GF2p.luc, a significant three- to sevenfold stimulation of FGF-2 promoter activity was detected in the hearts of two independent lines 6 h after intraperitoneal administration of phenylephrine (50 mg/kg). This increase was still apparent at 24 h but was not detected at 48 h posttreatment. Analysis of FGF-2 mRNA in normal mouse hearts revealed accumulation of the 6.1-kb transcript at 24 h. Control of local FGF-2 synthesis at the transcriptional level through adrenergic stimulation may be important in the response to injury as well as in the maintenance of a healthy myocardium.

basic fibroblast growth factor; rat fibroblast growth factor-2 gene; phenylephrine; gene transfer

FIBROBLAST GROWTH FACTOR (FGF)-2, also known as basic FGF, is a mitogenic and angiogenic protein that has been found in all tissues examined thus far (3, 16). The effects of FGF-2 are exerted through cell-surface, high-affinity tyrosine kinase receptors (FGFR) and low-affinity sites consisting of heparan sulfate proteoglycans (13, 20). Receptors for FGF-2 (FGFR-1) are present in the embryonic and adult heart (15, 17, 22, 23) and were shown to be essential for normal heart development (26). Although FGF-2 is present in the heart into adulthood (15, 23), its role in the postnatal heart is less clear. FGF-2 is found intracellularly as well as outside the cell, where it is able to exert its effect on cell-surface receptors. However, the mechanism for FGF-2 export is unclear because it contains no signal peptide (3). There is evidence for unconventional release of FGF-2 via a pathway independent of the endoplasmic reticulum-Golgi complex (25). Also, factors involved in facilitating or inhibiting the export of FGF-2 have been identified (10, 36). With regard to the postnatal heart, studies have shown that FGF-2 is released on contraction and that this can be regulated by increasing the heart rate and force of contraction by electrical or adrenergic stimulation (8, 18). The mechanism of release is reported to involve transient, nonlethal disruptions of the plasma membrane, a phenomenon that occurs in many tissues exposed to high levels of mechanical stress (24). Factors influencing FGF-2 release may also be expected to influence FGF-2 synthesis. The increases in FGF-2 mRNA as well as protein after more damaging types of tissue injury (5, 9, 30) raise the possibility that regulation at the level of transcription is a component of FGF-2 release associated with the transient remodeling of the membrane in the contracting heart.

Detection of FGF-2 mRNA appears to be more difficult than detection of protein, reflecting, presumably, low activity of the FGF-2 promoter and/or relatively unstable transcripts. This has necessitated the use of large amounts of RNA for blotting studies, often in conjunction with tumor cells overexpressing FGF-2 (4, 28). An alternative approach for transcriptional studies is the reporter gene assay. We recently cloned a 1.4-kb fragment of the rat genome containing −1 kb of FGF-2 upstream flanking DNA, including a promoter region, and reported the sequence for the −552/+252 domain (34). We have now examined an extended FGF-2 promoter region (−1,058/+54) for a response to adrenergic stimulation both in vitro, using the luciferase reporter gene for transient transfection of neonatal rat cardiac myocytes, and in vivo, through the generation of transgenic mice expressing the hybrid FGF-2/luciferase gene. We have also completed the sequencing of the 1.4-kb clone and examined it for the presence of adrenergic responsive DNA elements. Our data indicate that FGF-2 promoter activity is stimulated both in vitro and in vivo after adrenergic stimulation, and these results correlate with increases in endogenous FGF-2 RNA accumulation. Characterization of this response in vitro through the use of an α1-selective antagonist, prazosin, suggests that the majority of this effect is mediated through the α1-adrenergic pathway, but it does not appear to be dependent on either Ca2+ influx or myocyte contraction. These results are discussed in relation to a role for...
FGF-2 in maintaining a healthy mycardium during postnatal development and in adulthood.

MATERIALS AND METHODS

Cell culture and tissue extracts. Neonatal rat cardiomyocyte cultures were prepared essentially as previously described (35). Briefly, ventricles from rat pups (at 1–36 h after birth) were dissected, and the cells were dissociated in a spinner flask using a combination of trypsin (GIBCO BRL, Burlington, ON, Canada) and DNase I (Sigma-Aldrich, Oakville, ON, Canada). Myocytes were then separated from nonmuscle cells on a discontinuous Percoll gradient and plated on collagen-coated plates at a density of 1 x 10^6 cells per 35-mm dish. Cells were initially plated in Ham's F-10 medium containing 10% fetal bovine serum (FBS), 10% horse serum, antibiotic (1,000 U/ml penicillin, 1 mg/ml streptomycin), and CaCl_2 supplemented to 1.05 mM. All cultureware was purchased from Corning (Fisher Scientific, Nepean, ON, Canada), and all media and culture reagents were from GIBCO BRL.

Sequencing. Isolation of the genomic clone lambdaFGF2-c4 from a Sprague-Dawley rat testis genomic library, which included a 1.4-kb BamH I fragment (B2) containing FGF-2 coding sequences and upstream flanking DNA, including a promoter region, was described previously (34). The 1.4-kb B2 fragment was subcloned into pUC119, and nucleotide sequence was determined by the dideoxy method using a Promega Fentomole Sequencing Kit (Fisher Scientific). The sequence was analyzed for consensus binding sites for known transcription factors using TFSEARCH (Y. Akiyama, Kyoto University, Kyoto, Japan).

Plasmids and constructs. The hybrid genes −1058FGF-p.luc, −911FGFp.luc, and −313FGFp.luc, containing fragments of the rat FGF-2 gene fused upstream of a promoterless firefly luciferase gene (p.luc, contained in the vector pXP1) (29) were described previously (34). A 250-bp fragment of the myosin light chain-2 (MLC-2) promoter cloned upstream of luciferase was described previously (14).

RNA analyses. Total RNA was isolated from transfected rat cardiomyocyte cultures or mouse hearts using guanidine isothiocyanate (7). Total RNA was denatured with formaldehyde and resolved by electrophoresis through a 1.0% agarose gel. The RNA was blotted to nitrocellulose, probed with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (9), and visualized by autoradiography. The rat FGF-2 fragment corre-

RESULTS

FGF-2 RNA levels and promoter activity are stimulated by norepinephrine. Neonatal rat cardiomyocytes were isolated and treated without (control) or with 0.01 mM norepinephrine or norepinephrine with an α-adrenergic antagonist (0.01 mM prazosin) for 6 h to assess any effect on FGF-2 RNA levels. RNA was separated by gel electrophoresis, blotted to nitrocellulose, probed with a radiolabeled fragment of the rat FGF-2 cDNA, and visualized by autoradiography (Fig. 1A). The 28S RNA band seen with ethidium bromide staining before
transfer to nitrocellulose is also shown to allow a comparison of RNA levels (Fig. 1A). Although not as evident because of a discrepancy in loading, the 6.1-kb FGF-2 transcript level was increased after norepinephrine treatment for 6 h. Consistent with this observation, the 6.1-kb FGF-2 transcript was reduced and barely detectable after treatment with norepinephrine and the α1-specific antagonist prazosin.

To demonstrate control at the level of transcription, the FGF-2 promoter itself was then tested for adrenergic responsiveness. Neonatal rat cardiac myocytes, transiently transfected with a hybrid firefly luciferase gene directed by 1,112 bp (positions 1,058 to 1,54) of FGF-2 5′-flanking DNA (21058FGFp.luc), were treated with 0.01 mM norepinephrine in the absence or presence of 0.01 mM prazosin or a β-adrenergic antagonist (0.01 mM atenolol). The results are shown in Fig. 1B. Norepinephrine evoked a 2.5-fold increase in 21058FGFp.luc activity (expressed per ng protein) after 6 h of stimulation (P < 0.0001). This effect was completely abolished in the presence of prazosin. In contrast, a slight, but not significant, decrease in response to norepinephrine treatment was observed in the presence of atenolol.

A putative phenylephrine-responsive element is present in upstream FGF-2 flanking DNA. The complete sequence of a 1,389-bp genomic fragment containing rat FGF-2 5′-flanking DNA is shown in Fig. 2. This corresponds to nucleotide positions −1,058 through −331 based on the primary transcription start site (+1) described for the brain (34). Analysis of these sequences revealed two copies, in tandem, of putative phenylephrine-responsive elements (PEREs). These sequences (5′-AGGGAGGG-3′), located at nucleotide positions −780 and −761, were identified on the basis of their high degree of similarity to sequences present in the human skeletal actin (5′-AGGGAGGG-3′) and rat atrial natriuretic factor (ANF) promoters (5′-GGGGAGGG-3′) that have been implicated in the response to α1-specific adrenergic activation by phenylephrine (1). In the latter case, these sequences were shown to bind a specific protein complex and confer phenylephrine responsiveness (1). Consensus binding sites for known transcription factors identified in the FGF-2 sequences are also shown in Fig. 2.
FGF-2 RNA levels and promoter activity are increased by phenylephrine, but this effect appears to be independent of putative PEREs. To initiate a characterization of the putative PEREs in the FGF-2 DNA, conditions were established for phenylephrine stimulation of endogenous FGF-2 RNA levels. Neonatal rat cardiac myocytes were isolated and treated with the \( \alpha_1 \)-adrenergic agonist phenylephrine for 48 h (1) and then assessed by RNA blotting. An increase in the 6.1-kb FGF-2 transcript was detected with the FGF-2 cDNA probe after phenylephrine treatment for 48 h (Fig. 3A). The GAPDH and 28S RNA transcripts were also assessed as controls for RNA loading, and the results are included for comparison.

Transient gene transfer using truncated hybrid FGF-2/luciferase genes was used to assess the effect of phenylephrine on FGF-2 promoter activity as well as the involvement of the putative PEREs on any response observed. Convenient restriction endonucleases were used to generate 5′-deleted fragments of FGF-2 upstream sequences and produce basal FGF-2 promoter activity to norepinephrine-stimulated luciferase activity (Fig. 4A). Adrenergic stimulation of FGF-2 promoter activity was not affected by contraction arrest or Ca\(^{2+}\) influx. In an effort to investigate the role of contraction in the response of basal FGF-2 promoter activity to norepinephrine, cardiomyocytes transiently transfected with −1058FGFp.luc were treated with 0.01 mM norepinephrine in the presence of either KCl or 2,3-butanedione monoxime. Stimulation with 0.01 mM norepinephrine in the presence of either KCl or 2,3-butanedione monoxime caused a visible arrest of contraction of the cardiac myocytes during the 6-h incubation period but did not have a significant effect on norepinephrine-stimulated luciferase activity (Fig. 4A).

To further assess a role for \( \alpha_1 \) in the response of basal FGF-2 promoter activity to \( \alpha_1 \)-specific adrenergic stimulation, neonatal cardiac myocytes transfected with −1058FGFp.luc were treated with 0.1 mM phenylephrine for 48 h in the presence or absence of 1.0 \( \mu \)M nifedipine (Fig. 4B). Nifedipine arrested contraction, but no significant effect on luciferase activity was detected when compared with cells treated with phenylephrine alone.

Phenylephrine treatment increased −1058FGFp.luc transgene expression in the heart. Two independent transgenic mouse lines (P300 and P66) expressing FGF-2 RNA levels and promoter activity are increased by phenylephrine, but this effect appears to be independent of putative PEREs. To initiate a characterization of the putative PEREs in the FGF-2 DNA, conditions were established for phenylephrine stimulation of endogenous FGF-2 RNA levels. Neonatal rat cardiac myocytes were isolated and treated with the \( \alpha_1 \)-adrenergic agonist phenylephrine for 48 h (1) and then assessed by RNA blotting. An increase in the 6.1-kb FGF-2 transcript was detected with the FGF-2 cDNA probe after phenylephrine treatment for 48 h (Fig. 3A). The GAPDH and 28S RNA transcripts were also assessed as controls for RNA loading, and the results are included for comparison.

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whether the FGF-2 promoter responds to α2-adrenergic stimulation in the heart in vivo, adult mice (8–10 wks old) from the P300 line were injected intraperitoneally with 50 mg/kg phenylephrine and then euthanized 6, 24, or 48 h after injection. The hearts were removed, and luciferase activity per microgram of protein was determined (Fig. 5B). A significant 3.7-fold increase in luciferase gene expression compared with that in mice injected with saline alone was observed 6 h after phenylephrine treatment (P < 0.0005, n = 6). At 24 h, the difference, although not quite significant (P = 0.057), remained at 3.6-fold but was lost at 48 h. No significant changes in luciferase activity were observed in saline-injected animals between 6 and 48 h after injection (not shown). The phenylephrine response observed was confirmed in the P66 line, in which the difference at 6 h after phenylephrine administration was 6.9-fold (P < 0.005, n = 4). A parallel assessment of endogenous mouse heart FGF-2 RNA levels at each time point after phenylephrine treatment was done by RNA blotting (Fig. 5C). The level of endogenous mouse 6.1-kb FGF-2 transcript was increased at 24 h but was decreased again by 48 h after administration of phenylephrine. A second FGF-2 transcript of 3.6 kb was also observed in mouse preparations and showed the same pattern of response as the 6.1-kb mRNA. This 3.6-kb transcript was not seen in rat RNA preparations (Figs. 1 and 3).

**DISCUSSION**

Previously it was reported that FGF-2 is released from adult rat heart on contraction and that both release and contraction can be increased through adrenergic stimulation (8, 18). We have used RNA blotting and gene transfer to demonstrate that FGF-2 is under positive transcriptional control in the heart via the α-adrenergic pathway (Figs. 1–5). Norepinephrine is a naturally occurring catecholamine that acts through both α- and β-adrenoceptors (41). Endogenous FGF-2 RNA levels and transfected rat FGF-2 promoter (−1,058/+54) activity were both increased in neonatal rat cardiac myocytes after treatment for 6 h with norepinephrine (Fig. 1). This response was completely blocked with the α1-selective antagonist prazosin. Also, endogenous FGF-2 RNA accumulation and transfected FGF-2 promoter activity were stimulated in response to treatment with the α2-specific agonist phenylephrine (Fig. 3). Although β-adrenergic signaling was implicated in FGF-2 release from cardiac myocytes (8), we were unable to confirm a direct effect of β-receptor activation on synthesis at the transcriptional level using the −1058FGFp.luc gene. The apparent decrease in FGF-2 promoter activity seen after atenolol addition to norepi-
neprinone-treated cells was not quite significant (P = 0.07). Of course, this does not rule out the possibility that β-stimulation exerts its effect elsewhere in the synthetic pathway or that the 1,058 bp of FGF-2 5′-flanking DNA used were insufficient to respond to a β-adrenergic stimulus. Regardless, our data with phenylephrine as well as norepinephrine, in the presence or absence of prazosin, strongly implicate α₁-adrenoceptors in the regulation of FGF-2 synthesis in cardiac myocytes.

The rat genomic clone containing FGF-2 5′-flanking DNA includes promoter elements, which suggests that a wide variety of mechanisms may control FGF-2 transcription (Fig. 2). Like its human homologue, the rat FGF-2 gene promoter does not contain typical TATA or CAAT boxes (34, 38, 42). Instead, activation of transcription may involve binding of the promoter-specific factor Sp1 or early growth response protein-1 (Egr-1), because binding sites for these proteins are contained in a GC-rich region associated with the location of transcription initiation sites (2, 34). A search for known transcription factor consensus binding sites revealed putative binding sites (Fig. 2) for homeodomain- or basic helix-loop-helix domain-containing proteins, zinc finger proteins, and GATA proteins, and a 6-bp thyroid hormone-responsive element is present that has been described as a half-site for the thyroid hormone receptor (19). The ability of the −1058FGF-p.luc gene to respond to phenylephrine in the same positive manner as observed with the endogenous FGF-2 gene suggested that the genetic information contained within the region −1,058/+54 of the FGF-2 gene is sufficient for this response. Two tandem 8-bp elements (5′-AGGGAGGG-3′), which appear closely related to the reported PERE in the ANF promoter (5′-GGGGAGGG-3′) (1) were found within this region. Clearly, these elements were candidates for conferring α₁-adrenergic responsiveness on the FGF-2 promoter.

Although the activity of the FGF-2 promoter used in these studies was increased by both norepinephrine and phenylephrine, deletion of the putative PEREs had no effect on this response (Fig. 3B), suggesting that they do not play an essential role in α₁-adrenergic stimulation of the rat FGF-2 promoter. In the case of ANF, Spenkle et al. (40) reported that the PERE (referred to as an Sp-1-like element by these authors) was by itself responsible for less than threefold phenylephrine induction but that inclusion of an upstream serum-response element (SRE) increased the response to a level of 5.3–7.4-fold. Responses of ANF and MLC-2 promoters to phenylephrine were attributed in part to elements with an A/T-rich core (12, 40, 44, 45). In this context, it is interesting to note that an A/T-rich sequence is contained at nucleotide position −113/−108 (5′-TTTTAAA-3′) in the rat FGF-2 promoter (Fig. 2) and is identical to the core sequence of the SRE in the ANF promoter (40). These A/T-rich sequences are also conserved in the human FGF-2 gene (34, 38). Additional sequences conserved between the human and rat FGF-2 promoters are binding sites for Egr-1 (2, 38). Egr-1 binds to the consensus sequence 5′-GGGGAGGG-3′ (Fig. 2) and was shown to regulate FGF-2 transcription in astrocytes (2). Because protein kinase C (PKC) activation is known to upregulate Egr-1 in multiple cell types (6, 27, 37, 43), and because α₁-adrenergic-receptor effects are mediated through PKC (41), the Egr-1 elements might also be involved in the observed response of the FGF-2 promoter to phenylephrine treatment.

Although cardiac myocyte contraction can be stimulated through both α₁- and β-adrenoceptors (8, 41), our data indicate that adrenergic stimulation of FGF-2 promoter activity is not dependent on stimulation of contraction. Arrest of contraction using high extracellular KCl or 2,3-butanedione monoxime did not interfere with the increase in FGF-2 promoter activity after norepinephrine treatment (Fig. 4A). Interestingly, when KCl was used alone, a significant 1.5-fold increase in FGF-2 promoter activity was observed (not shown), raising the possibility that changes in intracellular Ca²⁺ may be a component of the response to adrenergic stimulation. The slow, permanent depolarization of the sarcolemma by high extracellular KCl would cause a corresponding permanent increase in intracellular Ca²⁺. However, blocking of the major (L-type) Ca²⁺ channels in cardiac myocytes with nifedipine had no effect on phenylephrine-induced FGF-2 promoter activity (Fig. 4B). This does not rule out the possible contribution of Ca²⁺ via minor channel types and intracellular stores.

In addition, we used transgenic mice to show that FGF-2 synthesis can be regulated at the transcriptional level by α₁-adrenergic stimulation in vivo (Fig. 5). The rat FGF-2 promoter, like its human counterpart, possesses properties associated with a housekeeping gene, and its product is found in all tissues studied (3, 16, 28, 42). This was reflected in the detection of luciferase activity in both brain and heart of transgenic mice (Fig. 5A). The increase in endogenous mouse FGF-2 RNA levels observed 24 h after administration of phenylephrine is consistent with the stimulation of FGF-2 promoter activity observed at this time and preceding this event at 6 h (Fig. 5, B and C). The loss of increased FGF-2 promoter activity as well as the decrease in FGF-2 RNA levels at 48 h likely reflects metabolism and clearance of the phenylephrine and a corresponding reduction in adrenergic stimulation. Regardless, the accumulation of FGF-2 RNA and, more specifically, the stimulation of FGF-2 promoter activity via α₁-adrenoceptors in the transgenic mice indicate a role for this regulatory pathway in vivo. Furthermore, the transfection and transgenic mouse data (Figs. 3 and 5) suggest that the −1,058/+54 region of the rat FGF-2 gene contains sufficient information to allow adrenergic regulation of the FGF-2 promoter in vitro and in vivo.

FGF-2 has many properties that make it a candidate for maintaining a healthy myocardium as well as offering protection against injury. FGF-2 enhances de novo angiogenesis (21) and promotes cell survival (11). Also, FGF-2 was shown to be protective against free radical damage in isolated cardiac myocytes (31), and, as shown with the use of a model of ischemia-reperfusion, FGF-2 improved recovery of function of the intact myocardium (31, 32). Clearly, an increase in
chronic levels of endogenous FGF-2 in the heart would potentially limit the extent of damage and improve recovery from an ischemic episode. Our data show that endogenous FGF-2 synthesis in the heart can be regulated at the transcriptional level by adrenergic stimulation.

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