Endothelin-1 induces expression of fetal genes through the interleukin-6 family of cytokines in cardiac myocytes

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Abstract We here examined the role of the interleukin-6 (IL-6) family of cytokines in endothelin-1 (ET-1)-induced hypertrophic responses using cultured cardiac myocytes of neonatal rats. ET-1 induced expression of IL-6 and leukemia inhibitory factor (LIF) genes. ET-1-induced LIF gene expression was abolished by inhibition of protein kinase C activity. ET-1 activated the promoter of atrial natriuretic peptide and β-type myosin heavy chain genes through the tyrosine kinase pathway and IL-6 receptor gp130. These results suggest that the IL-6 family of cytokines mediates ET-1-induced expression of some fetal genes in cardiac myocytes.

Key words: Endothelin-1; Interleukin-6; Leukemia inhibitory factor; Atrial natriuretic peptide; Brain natriuretic peptide; β-Type myosin heavy chain

1. Introduction

Clinical studies have demonstrated that cardiac hypertrophy is not only an adaptational state before cardiac failure, but also an independent risk factor for cardiac morbidity and mortality [1]. Thus, it has become even more important to determine the molecular mechanism of cardiac hypertrophy. Recent studies have indicated that cardiac hypertrophy is induced by mechanical stress [2] and neurohumoral factors such as angiotensin II (Ang II) [3], endothelin-1 (ET-1) [4], phenylephrine [5] and the interleukin-6 (IL-6) family of cytokines [6,7] in cardiac myocytes.

ET-1 has been reported to be not only a vasoconstrictor, but also a potent hypertrophy-promoting factor in a variety of mammalian cells including vascular smooth muscle cells [8], cardiomyocytes [9] and fibroblasts [10]. We have reported that mechanical stress induces hypertrophic responses such as activation of mitogen-activated protein kinase (MAPK) and Raf-1 kinases, expression of specific genes and an increase in protein synthesis in cardiac myocytes partly through ETA receptors [4]. Furthermore, it has been shown that ET-1 induces expression of proto-oncogenes [9,11], the brain natriuretic peptide (BNP) gene [12] and muscle specific genes such as myosin light chain 2, skeletal α-actin and troponin I [9]. These results suggest that ET-1 is produced from cardiomyocytes and plays an important role in the development of cardiac hypertrophy including induction of fetal genes. However, it remains to be determined how ET-1 induces fetal gene expression in cardiomyocytes.

The IL-6 family of cytokines, including IL-6, IL-11, leukemia inhibitory factor (LIF), cardiotrophin-1 (CT-1) and ciliary neurotrophic factor, has been reported to induce cardiomyocyte hypertrophy through gp130, a common β-receptor [13]. These IL-6-related cytokines activate Janus kinase (JAK), which evokes two signaling pathways, MAPK and signal transducer and activator of transcription (STAT) [13]. Furthermore, it has been shown that the IL-6 family of cytokines induces expression of *c-fos* and atrial natriuretic peptide (ANP) genes, followed by an increase in protein synthesis in cardiomyocytes [6,14].

To elucidate the relation between ET-1 and the IL-6 family of cytokines, we here examined the possibility that the IL-6 family of cytokines is involved in ET-1-induced expression of fetal genes in cultured cardiomyocytes of neonatal rats.

2. Materials and methods

2.1. Cell culture

Primary cultures of cardiac myocytes were prepared from the ventricles of 1 day old Wistar rats as described previously [2]. Cells were plated onto plastic culture dishes at a field density of 1×10^5 cells/cm^2.

2.2. RNA isolation and reverse transcription followed by polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from cardiac myocytes by using RNA STAT-60 (TEL-TEST B) according to the manufacturer's instructions. RNA was treated with DNAse to eliminate any contamination of genomic DNA. Rat IL-6, LIF, CT-1 and GAPDH mRNA were amplified by PCR using the following primers.

Rat IL-6, 5′-GACTGATGTTGTTGACAGCCACTGC-3′ for sense and 5′-TAGCCACTCCTTCTGTGACTCTAACT-3′ for antisense; rat LIF, 5′-AGTCAACTGGCTCAACTCAACG-3′ for sense and 5′-CTGGGCCAACACACTTATGACT-3′ for antisense; rat CT-1, 5′-GAGACCGTGCTGGCCGCTCTGG-3′ for sense and 5′-GGCGAGAGCAGAAGAAGAAAGAG-3′ for antisense; rat GAPDH, 5′-CCATGGAGAAGGCTGGGG-3′ for sense and 5′-CAAAGTTGTCATGGATGACC-3′ for antisense. The predicted sizes of the PCR-amplified products were 509 bp for IL-6, 441 bp for LIF, 188 bp for CT-1 and 175 bp for GAPDH. PCR was performed with a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT, USA) using various cycles. After amplification, products were analyzed by electrophoresis on a 2–3% agarose gel.

2.3. Transfection and reporter gene assay

0.5 μg of reporter plasmid with or without 1 ng of dominant negative mutant of gp130 plasmid (D.N. gp130, kindly provided by Dr T. Taga) [15] were transfected into cardiac myocytes cultured in a 35 mm dish. The cells were washed with PBS at 6 h after transfection and incubated with serum free DMEM for 24 h. Cells were then stimulated by ET-1 (10^{-7} M) or LIF (10^3 U/ml) and incubated for 48 h. The luciferase activities were measured by a luminometer (Luminoscan, Labsystems). All transfection experiments were performed three times.

2.4. Statistical analysis

All results are expressed as mean ± S.E.M. Multiple comparisons among three groups were carried out by two-way ANOVA and Fish-

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er's exact test for post hoc analyses. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. ET-1 induces expression of IL-6-related cytokine genes in cardiomyocytes

Since it has been reported that ET-1 regulates synthesis of IL-6 under inflammations in endothelial cells [16], bronchial epithelial cells [17] and leukocytes [18], we examined whether ET-1 induces expression of IL-6-related cytokine genes in cardiac myocytes by RT-PCR. Untreated cardiac myocytes did not express any IL-6-related cytokines. When ET-1 (10^{-7} M) was added to cultured cardiac myocytes, mRNA levels of IL-6 and LIF began to increase from 30 min and peaked at 60 min. The mRNA levels were decreased thereafter, but were still significantly higher than control levels at 120 min. ET-1 did not increase expression levels of the CT-1 gene in cardiac myocytes (Fig. 1).

3.2. ET-1-induced expression of IL-6-related cytokine genes is mediated by the PKC-dependent pathway

It has been reported that ET-1 activates phospholipase C, which causes protein kinase C (PKC) and an increase in Ca^{2+} in many cell types including cardiac myocytes [4,10]. Since activated PKC induces hypertrophic responses such as an increase in protein synthesis and reprogramming of gene expressions in cardiac myocytes [2,19], we examined whether ET-1 upregulates expression of IL-6-related cytokine genes through the PKC-dependent pathway in cardiac myocytes. 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (10^{-6} M) induced expression of the LIF gene by almost the same levels as with ET-1 (Fig. 2). ET-1-induced LIF gene expression was completely inhibited by pretreament with a PKC inhibitor, calphostin C (5×10^{-6} M) for 60 min or with TPA (10^{-7} M) for 24 h [19] (Fig. 2). We next examined the role of Ca^{2+} in expression of IL-6-related cytokine genes. Incubation with a Ca^{2+} ionophore, ionomycin (10^{-6} M), for 60 min, induced expression of IL-6 and LIF mRNA (Fig. 3). Although expression of CT-1 was observed in unstimulated control cells, expression levels of the CT-1 gene were not changed by ionomycin as well as

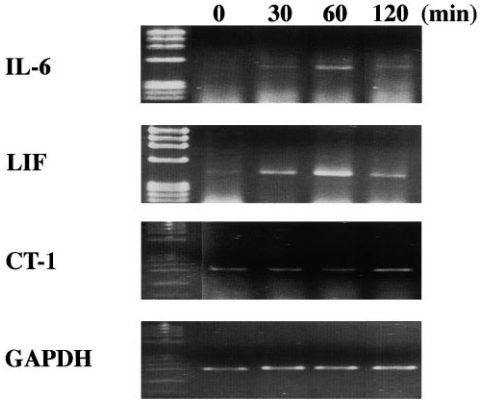

Fig. 1. Effects of ET-1 on IL-6, LIF and CT-1 gene expression in neonatal rat cardiomyocytes. Cells were incubated with ET-1 (10^{-7} M) for the indicated times. DNA amplified by RT-PCR was electrophoresed on 2–3% agarose gels and stained with ethidium bromide. GAPDH was also amplified from the same cDNA to show that equal amounts of cDNA were used in each lane.

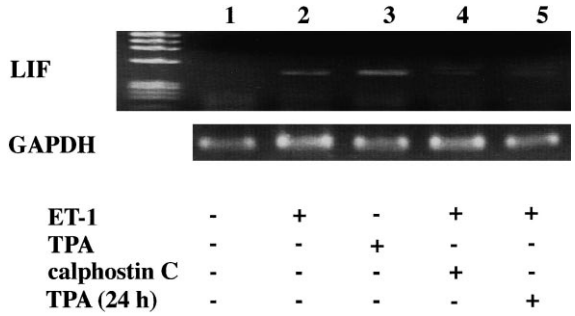

Fig. 2. The role of PKC in ET-1-induced LIF gene expression in cardiomyocytes. Cells were pretreated with calphostin C (5×10^{-6} M) for 30 min or TPA (10^{-7} M) for 24 h and stimulated by ET-1 or TPA. RT-PCR was performed and amplified DNA was subjected to gel electrophoresis. Lanes: 1, control; 2, ET-1 (10^{-7} M); 3, TPA (10^{-6} M); 4, ET-1 plus calphostin C (5×10^{-6} M) pretreatment for 30 min; 5, ET-1 plus TPA (10^{-7} M) pretreatment for 24 h.

ET-1 (data not shown). To examine whether Ca^{2+} is critically involved in ET-1-induced expression of IL-6-related cytokine genes, we pretreated cardiac myocytes with a calmodulin inhibitor, W-7 (10^{-5} M) [20], a Ca^{2+}/calmodulin-dependent protein (CaM) kinase II inhibitor, KN-93 (3×10^{-5} M) [21], a calcineurin inhibitor, cyclosporin A (5×10^{-6} M) [22], and extracellular Ca^{2+} chelator, EGTA (5×10^{-3} M). ET-1-induced expression of the LIF gene was not affected by these inhibitors except EGTA (Fig. 3), suggesting that PKC but not Ca^{2+} plays an important role in ET-1-induced expression of the LIF gene.

3.3. ET-1 activates ANP and β-type myosin heavy chain (β-MHC) promoters through tyrosine kinases

The hypertrophic responses in cardiac myocytes are characterized by the induction of a fetal gene program [23]. It has been reported that ET-1 and CT-1 induce expressions of fetal genes such as ANP and skeletal α-actin through PKC activation [9] and tyrosine kinases [6], respectively. To get insight into the relation between ET-1- and IL-6-related cytokines, we examined whether tyrosine kinases are also involved in ET-1-

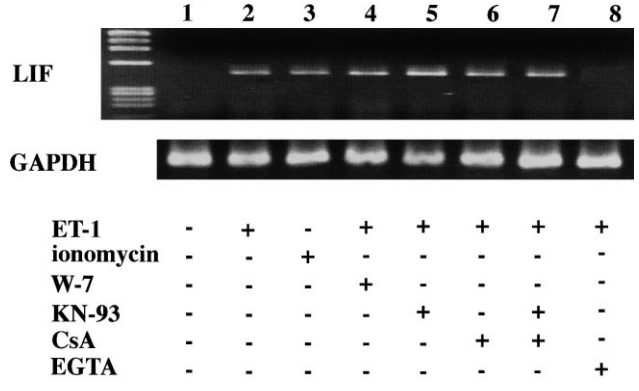

Fig. 3. The role of Ca^{2+} in ET-1-stimulated LIF gene expression. Cells were pre-incubated with W-7 (10^{-5} M), KN-93 (3×10^{-5} M), cyclosporin A (5×10^{-6} M) for 1–3 h or EGTA (5×10^{-3} M) for 1 min and stimulated by ET-1 or ionomycin for 60 min. RT-PCR was performed and amplified DNA was subjected to gel electrophoresis. Lanes: 1, control; 2, ET-1 (10^{-7} M); 3, ionomycin (10^{-6} M); 4, ET-1 plus W-7 (10^{-5} M) pretreatment; 5, ET-1 plus KN-93 (3×10^{-5} M) pretreatment; 6, ET-1 plus cyclosporin A (5×10^{-6} M) pretreatment; 7, ET-1 plus KN-93 and cyclosporin A pretreatment; 8, ET-1 plus EGTA (5×10^{-3} M) pretreatment.

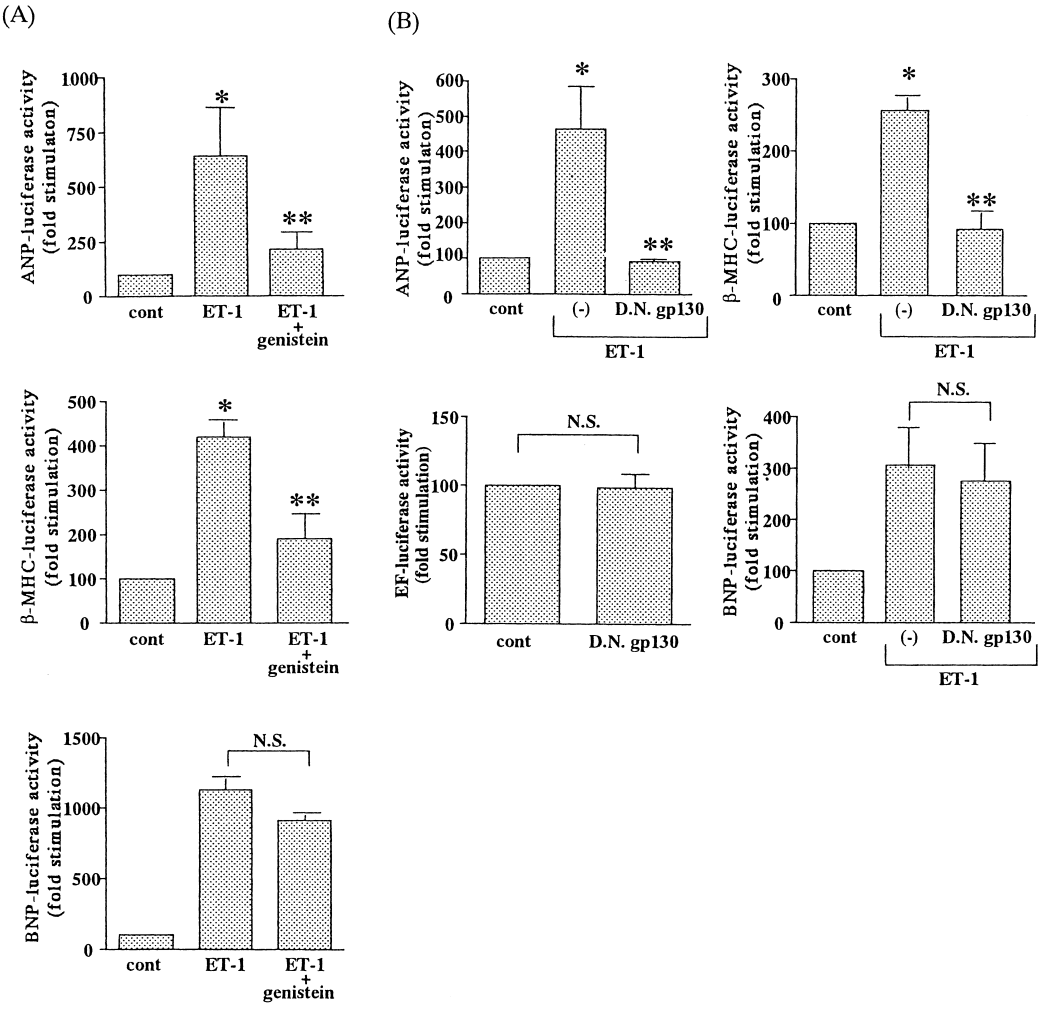

Fig. 4. The roles of tyrosine kinases and gp130 in ET-1-induced activation of ANP, BNP and β-MHC promoters. (A) Cells transfected with ANP, BNP or β-MHC promoter/luciferase reporter gene were stimulated by ET-1 (10^{-7} M) for 48 h with or without pretreatment of genistein (5×10^{-4} M) for 30 min. (B) ANP, β-MHC, BNP or EF promoter/luciferase was transfected into cardiomyocytes with or without the D.N. gp130 gene and then, cells were stimulated by ET-1 (10^{-7} M) for 48 h. Luciferase activity was normalized to *Renilla* luciferase activity for each sample and expressed as the fold increase relative to no drug treatment (= 100). Data show the mean ± S.E.M. of three independent assays. *, $P<0.05$ versus control. **, $P<0.05$ versus ET-1 treatment without D.N. gp130.

induced expression of cardiac fetal genes. Incubation with ET-1 (10^{-7} M) for 48 h increased ANP-, BNP- and β-MHC promoter activities by 11.3-, 6.4- and 4.2-fold as compaired with control levels, respectively. When cardiac myocytes were pre-incubated with a tyrosine kinase inhibitor, genistein (5×10^{-6} M) for 60 min, ET-1-induced activation of ANP and β-MHC genes was significantly suppressed. However, pretreatment with genistein did not affect the promoter activity of the BNP gene enhanced by ET-1 (Fig. 4A).

3.4. Overexpression of the dominant negative mutant of gp130 inhibits ANP and β-MHC promoter activation induced by ET-1

To clarify whether IL-6-related cytokines are involved in ET-1-induced fetal gene expressions, we transfected a dominant negative mutant of gp130 (D.N. gp130) with fetal gene promoters into cardiac myocytes. The transfection efficiency of luciferase reporter genes was not changed with or without D.N. gp130 (data not shown). ANP- and β-MHC-luciferase activities enhanced by ET-1 (10^{-7} M) were strongly inhibited by overexpression of D.N. gp130. In contrast, ET-1-induced activation of BNP and elongation factor (EF) promoters were not affected by overexpression of D.N. gp130 (Fig. 4B). These results indicate that gp130 is critically involved in ET-1-induced reprogramming of ANP and β-MHC gene expression in cardiac myocytes.

3.5. LIF activates ANP, BNP and β-MHC promoters

It has been reported that gp130 activation by exposure to LIF or CT-1 causes induction of ANP expression in cardiac myocytes [6,14]. Therefore, we finally examined whether LIF activates the promoter of BNP and β-MHC genes in cardiac myocytes. LIF (10^3 U/ml) increased for 48 h ANP, BNP and β-MHC luciferase activities by approximately 9.6-, 5.0- and 2.8-fold as compared with control levels, respectively (Fig. 5). These results indicate that LIF induces expression of BNP and β-MHC genes as well as the ANP gene in cardiac myocytes.

4. Discussion

A variety of growth and neurohumoral factors such as Ang

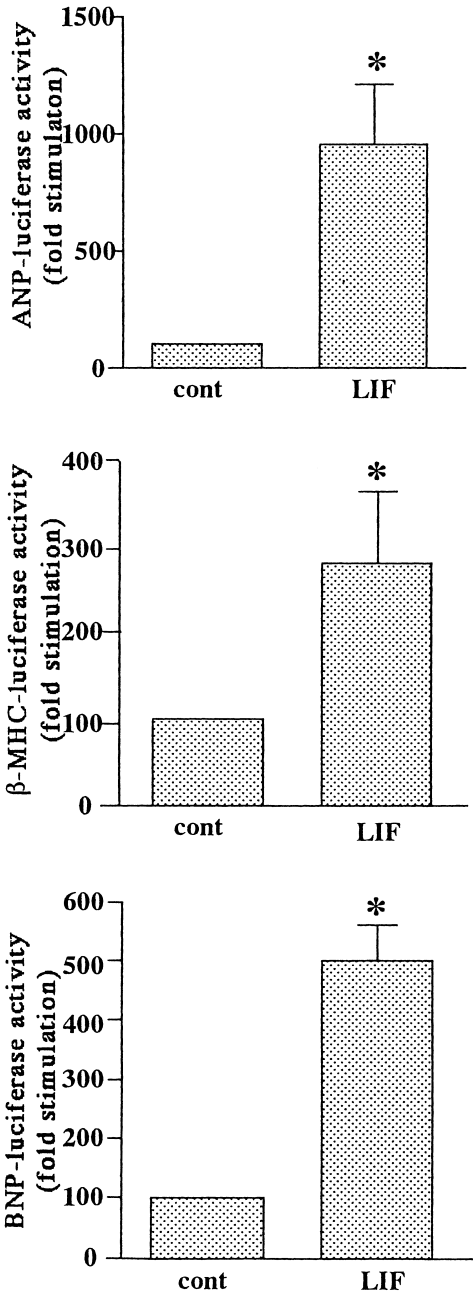

Fig. 5. Effect of LIF on ANP, BNP and β-MHC promoters. Cells were transfected with ANP, BNP, or β-MHC promoter/luciferase reporter gene and then stimulated by LIF (10^3 U/ml) for 48 h. Data show the mean ± S.E.M. of three independent assays. *, $P < 0.05$ versus control.

II [19], ET-1 [4] and α1-adrenergic agonist [5] have been reported to induce hypertrophic responses in cardiomyocytes. Recently, ET-1 has been reported to induce expression of IL-6 and LIF genes in rat endothelial cells [16] and mesangial cells [24] through an increase in intracellular Ca^{2+} levels. In the present study, ET-1 induced expression of IL-6 and LIF genes in cardiac myocytes. ET-1-induced LIF gene expression was completely suppressed by inhibition or down-regulation of PKC activity and TPA upregulated mRNA levels of IL-6 and LIF. Although ionomycin also induced expression of IL-6 and LIF genes, inhibition of calmodulin, CaM kinase II and calcineurin had no effect on expression of LIF mRNA by ET-1. These results suggest that PKC activation is important for ET-1-induced LIF mRNA expression in cardiac myocytes. Since some isoforms of PKC are activated in response to an increase in intracellular Ca^{2+} [25], ionomycin may induce expression of these cytokines through activation of PKC and ET-1-induced LIF gene expression may be inhibited by EGTA in cardiac myocytes. Further studies are required to elucidate which isoform of PKC is involved in ET-1-induced expression of these cytokines. CT-1 is also a member of the IL-6 cytokine family and has a wide range of growth and differentiation activities on many cell types including cardiac myocytes [6]. The present study demonstrated that although CT-1 was expressed in untreated cardiomyocytes, stimulation with ET-1 or ionomycin did not enhance mRNA levels of CT-1. Recently, it has been reported that the 5′-flanking region of the human CT-1 gene is isolated [26]. The regulatory mechanism of CT-1 may be different from that of IL-6 and LIF.

It has recently been reported that activation of tyrosine kinases increases expression of ANP, β-MHC and skeletal α-actin genes in rat cardiomyocytes [27] and that ET-1 rapidly enhances tyrosine phosphorylation in mesangial cells or vascular smooth muscle cells. We examined the possibillity that activation of tyrosine kinase is involved in ET-1-induced fetal gene expression. When cardiomyocytes were pretreated with a tyrosine kinase inhibitor, ET-1-induced expression of ANP and β-MHC genes, but not the BNP gene, was strongly reduced. Since ET-1 directly activates PKC but not tyrosine kinases in cardiac myocytes, we speculate that ET-1 induces the fetal gene program through other factors which activate tyrosine kinases.

It has been reported that IL-6-related cytokines activate the tyrosine kinase JAK through gp130 in cardiomyocytes [7]. Overexpression of D.N. gp130 markedly suppressed ET-1-induced activation of ANP and β-MHC promoter in cardiac myocytes and LIF activated promoters of ANP, BNP and β-MHC genes. Taken together, IL-6-related cytokines induced by ET-1 may activate tyrosine kinases through gp130, leading to activation of the fetal gene program during cardiac hypertrophy. However, ET-1-induced activation of the BNP promoter gene was not inhibited by either a tyrosine kinase inhibitor or overexpression of D.N. gp130. It has been reported that transcription of the BNP gene is rapidly increased in response to hemodynamic overload following heart failure and myocardial infarction [28]. ET-1 also rapidly increases expression levels of the BNP gene within 60 min in cultured rat cardiomyocytes [12], indicating that the BNP gene is one of the immediate early response genes. Furthermore, it has been reported that the ET-1-induced BNP gene expression is significantly suppressed by PKC inhibitors [12]. This result suggests that PKC activation is one of the proximal signaling pathways in the rapid induction of the BNP gene expression. Therefore, it is possible that ET-1 may induce expression of cardiac specific genes through two different pathways, one pathway depends on IL-6-related cytokines and the other pathway is directly activated by ET-1 in cardiac myocytes.

In summary, these studies suggest that IL-6-related cytokines are induced by ET-1 through the PKC pathway in cardiac myocytes and that ET-1-induced activation of the fetal gene program is mediated by the cytokines. It remains to be determined how these cytokines induce expression of fetal genes.

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