

Activation of nuclear factor- κ B is necessary for myotrophin-induced cardiac hypertrophy

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The transcription factor nuclear factor- κ B (NF- κ B) regulates expression of a variety of genes involved in immune responses, inflammation, proliferation, and programmed cell death (apoptosis). Here, we show that in rat neonatal ventricular cardiomyocytes, activation of NF- κ B is involved in the hypertrophic response induced by myotrophin, a hypertrophic activator identified from spontaneously hypertensive rat heart and cardiomyopathic human hearts. Myotrophin treatment stimulated NF- κ B nuclear translocation and transcriptional activity, accompanied by I κ B- α phosphorylation and degradation. Consistently, myotrophin-induced NF- κ B activation was enhanced by

wild-type I κ B kinase (IKK) β and abolished by the dominant-negative IKK β or a general PKC inhibitor, calphostin C. Importantly, myotrophin-induced expression of two hypertrophic genes (atrial natriuretic factor [ANF] and *c-myc*) and also enhanced protein synthesis were partially inhibited by a potent NF- κ B inhibitor, pyrrolidine dithio-carbamate (PDTC), and calphostin C. Expression of the dominant-negative form of I κ B- α or IKK β also partially inhibited the transcriptional activity of ANF induced by myotrophin. These findings suggest that the PKC–IKK–NF- κ B pathway may play a critical role in mediating the myotrophin-induced hypertrophic response in cardiomyocytes.

Introduction

Cardiac hypertrophy is a major risk for heart failure and sudden death (Levy et al., 1990). It has been shown that in animals or cultured neonatal rat cardiomyocytes, cardiac hypertrophy can be induced by a combination of mechanical forces and neurohumoral stimuli, leading to an increase in cardiac protein synthesis and cell growth through various signaling pathways (Morgan and Baker, 1991; Glennon et al., 1995; Kijima et al., 1996; Dostal et al., 1997; Hefti et al., 1997).

In cultured neonatal rat cardiomyocytes, both mechanical and biochemical stimuli cause changes in gene expression that closely parallel the hypertrophied heart in vivo. This includes the sequential activation of different proto-oncogenes and hypertrophic marker genes such as atrial natriuretic factor (ANF)* and β -myosin heavy chain (Izumo et al., 1987, 1988; Iwaki et al., 1990; Chien et al., 1991; Gammage and Franklyn, 1991; Sadoshima et al., 1992; Takahashi et al.,

1992; Sadoshima and Izumo, 1993; Liang et al., 1997). The signaling mechanism of the hypertrophic response remains incompletely understood. Significant efforts have been directed toward the identification of factor(s) involved in the hypertrophic process. Myotrophin, a 12-kD soluble protein, was originally identified by Sen et al. (1990) from spontaneously hypertrophied rat hearts and cardiomyopathic human hearts (Sen et al., 1990; Sil et al., 1993). It has been shown that myotrophin can initiate hypertrophy, with increased contractile protein synthesis, induction of proto-oncogenes and hypertrophic marker genes, and activation of PKC associated with an increase in surface area of the myocyte and the appearance of organized myofibrils within 48 h (Mukherjee et al., 1993; Sil et al., 1993, 1995, 1998). In addition, myotrophin expression is elevated under different hypertrophic conditions (Mukherjee et al., 1993). The gene coding for myotrophin has been cloned (Sivasubramanian et al., 1996) and structural/functional motif analysis revealed that it contains ankyrin repeats and putative phosphorylation sites for PKC and casein kinase II. Recently, we reported that myotrophin can bind to κ B DNA sequence, and by using various parameters, both in vitro and in vivo systems, we have shown that myotrophin- κ B DNA interaction is an important step in the initiation process of cardiac hypertrophy (Gupta and Sen, 2002).

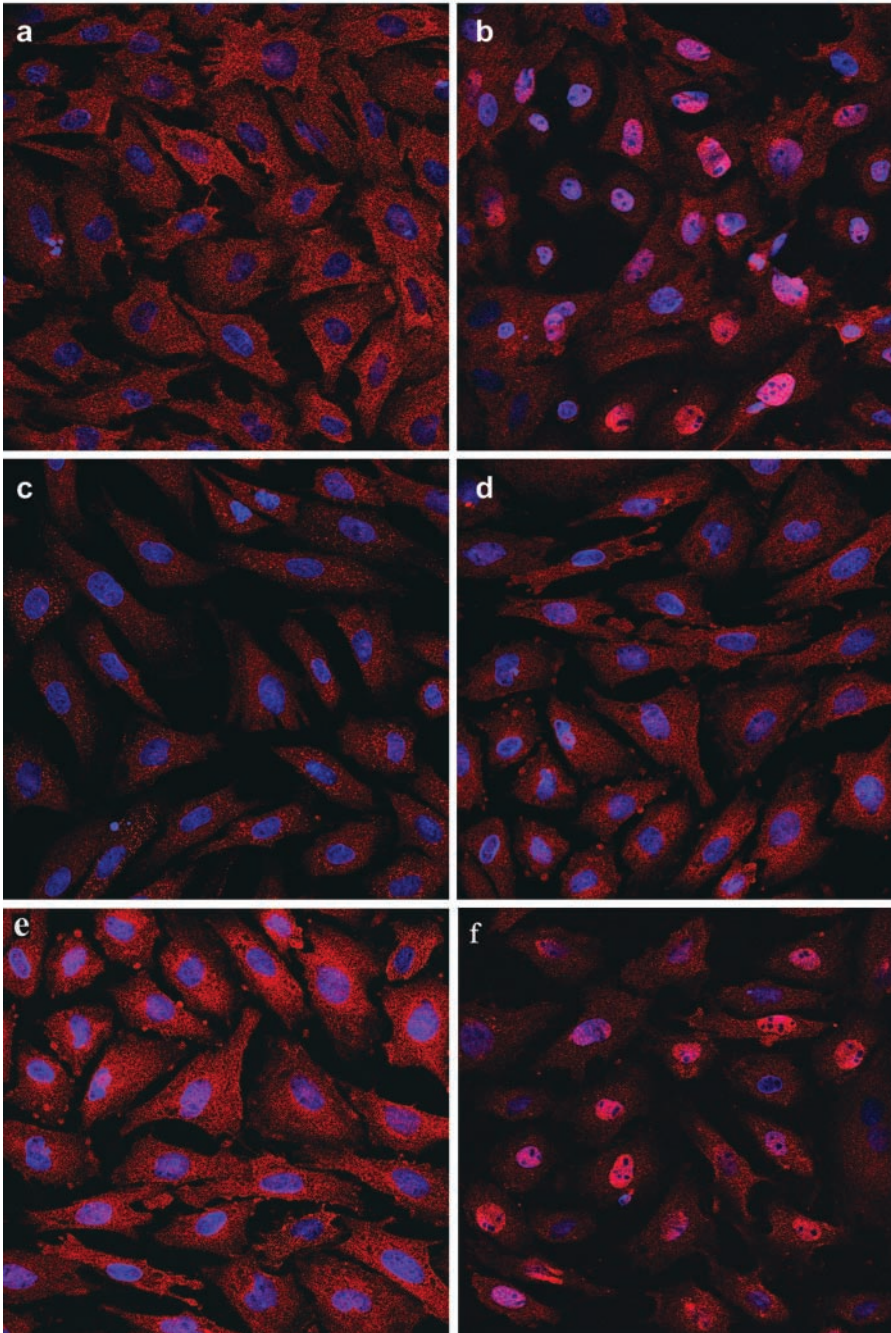
Nuclear factor- κ B (NF- κ B) was first identified as a B cell nuclear factor and was given its name on the basis of its abil-

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*Abbreviations used in this paper: ANF, atrial natriuretic factor; EMSA, electrophoretic mobility shift analysis; IKK, I κ B kinase; Luc, luciferase; NF- κ B, nuclear factor- κ B.

Key words: myotrophin; NF- κ B; PKC; cardiac hypertrophy; signal transduction

A



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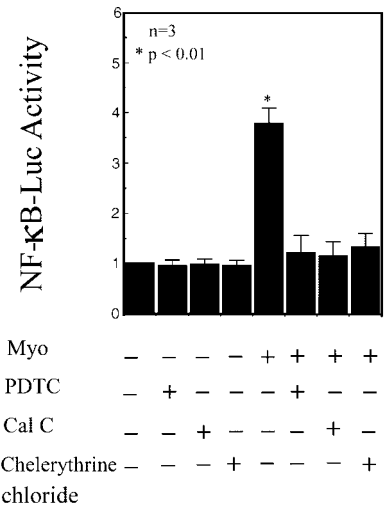


Figure 1. Stimulation of NF-κB activity by myotrophin in primary neonatal rat cardiomyocytes. (A) Neonatal myocytes were grown on coverslips, serum starved for 24 h, and treated with myotrophin (b) or left untreated (a). Cells were treated with calphostin C and PDTC before myotrophin treatment (c and d). Cells were also pretreated with TNFα (f) or left untreated (e) and used as a control. Cells were fixed, blocked, and stained with p65 mAb followed by anti-mouse Texas red (Molecular probe). The nuclear translocation of NF-κB p65 subunit was visualized by confocal microscopy. (B) Primary neonatal myocytes were transfected with 2X NF-κB reporter plasmid (1 μg/well) and treated with myotrophin for 24 h or left untreated. Also, cells were transfected with empty vector and treated with PDTC, calphostin C, and chelerythrine chloride before myotrophin treatment. Cells were harvested and the Luc activity was determined and normalized to the protein content of each extract. Luc activity expressed by cells transfected with empty vector was given an arbitrary value of 1. The results are presented as the mean ± SEM and represent three individual experiments. P < 0.01 compared with the control (untreated).

ity to bind to an enhancer of the immunoglobulin κ light chain gene (Sen and Baltimore, 1986). Since then, NF-κB has been identified as a ubiquitous inducible transcription factor that activates a number of genes (Baeuerle and Baltimore, 1996; Baldwin, 1996; May and Ghosh, 1998). The IκB kinase (IKK) complex is a multiprotein complex with a molecular weight of 700–900 kD (Zandi et al., 1997). The IKK complex contains two catalytic subunits, IKKα and IKKβ (also known as IKK-1 and IKK-2, respectively) (Zandi et al., 1997), and an essential regulatory subunit, NEMO/IKKγ/IKKAP1 (Brockman et al., 1995; Zandi et al., 1997; Rothwarf et al., 1998; Yamaoka et al., 1998). IKK is activated by protein phosphorylation induced by a variety of

stimuli, from proinflammatory cytokines to physical stress (Chen et al., 1996; DiDonato et al., 1997; Regnier et al., 1997; Woronicz et al., 1997; Zandi et al., 1997, 1998; Zandi and Karin, 1999). Once activated, IKK phosphorylates IκBs, a group of cytoplasmic NF-κB inhibitors, on specific serine residues (Ser32 and Ser36 in IκBα, and Ser19 and Ser23 in IκBβ), triggering their ubiquitination and subsequent degradation by the 26S proteasome (Traenckner et al., 1994; Thanos and Maniatis, 1995; Whiteside, 1995). Degradation of IκB un masks NF-κB's nuclear translocation signal, which allows NF-κB to translocate into the nucleus, where it stimulates transcription of specific target genes that are involved in immune responses, inflammation, viral infec-

tion, and cell survival (Brown et al., 1995; Chen et al., 1995; Finco and Baldwin, 1995; Li et al., 1999). NF- κ B has been shown to play a pivotal role in the induction of gene expression during ischemic adaptation and heart failure (Huarte and Stutz, 1992; Das et al., 1995; Maulik et al., 1998; Ritchie, 1998; Chen et al., 1999; Xuan et al., 1999; De Martin et al., 2000; Li et al., 2001). The potential role of hypertrophic agonist-mediated NF- κ B activation in neonatal myocytes has also been reported (Peng et al., 1995; Brasier et al., 2000; Rouet-Benzineb et al., 2000). Recently, it has been shown that in cultured neonatal cardiomyocytes, activation of NF- κ B by various hypertrophic agonists (Purcell et al., 2001b) is necessary for the hypertrophic response. Because myotrophin can activate PKC in neonatal cardiomyocytes (Sil et al., 1998) and PKC is involved in NF- κ B activation (Goodfriend et al., 1996), we explored NF- κ B activation in response to myotrophin in neonatal cardiomyocytes and its involvement in hypertrophic gene expression.

Results

Stimulation of NF- κ B activity by myotrophin in neonatal cardiac myocytes

To evaluate the role of NF- κ B in regulating hypertrophic growth in cardiomyocytes, we tested whether myotrophin can stimulate NF- κ B. Neonatal myocytes were grown on laminin-coated coverslips, serum starved, and treated with myotrophin. Myotrophin stimulated the nuclear translocation of p65 (Red), a member of the NF- κ B family, as measured by indirect immunofluorescence assays (Fig. 1 A, b). Pyrrolidine dithio-carbamate (PDTC), an inhibitor of NF- κ B, and calphostin C, a PKC inhibitor, blocked myotrophin-induced NF- κ B translocation (Fig. 1 A, c and d). The nuclear translocation of p65 was detected 30 min after treatment with myotrophin, with kinetics similar to TNF- α -treated cells (Fig. 1 A, e and f).

To determine whether myotrophin could stimulate NF- κ B transcriptional activity, neonatal myocytes were grown in six-well plates, transiently transfected with the 2X NF- κ B-luciferase (Luc) reporter gene, serum starved, and treated with myotrophin. Myotrophin significantly stimulated NF- κ B transcriptional activity 3.1-fold (Fig. 1 B), as measured by activation of the NF- κ B-Luc reporter gene. Cells pretreated with calphostin C, chelerythrine chloride, and PDTC for 1 h before treatment with myotrophin had a partial inhibition in NF- κ B transcriptional activity of 1.16-, 1.34-, and 1.22-fold, respectively (Fig. 1 B). Thus, NF- κ B appears to be a novel target of myotrophin in neonatal myocytes and its activation appears to be PKC dependent.

Myotrophin induces NF- κ B binding activity in neonatal myocytes and is PKC dependent

To investigate whether myotrophin could stimulate NF- κ B binding activity in neonatal myocytes, an electrophoretic mobility shift analysis (EMSA) was performed. Nuclear extracts of myotrophin-treated cells were prepared and 32 P-labeled NF- κ B DNA was used as a probe. Myotrophin treatment stimulated NF- κ B binding activity as shown in Fig. 2 A. The specific binding of NF- κ B in the myotrophin-treated

neonatal myocytes was confirmed by the addition of 100-fold molar excess of unlabeled NF- κ B competitor DNA into the EMSA reaction. Fig. 2 A shows that the unlabeled NF- κ B DNA competed for binding in nuclear extracts prepared from myotrophin-treated neonatal myocytes. The presence of NF- κ B in the protein complex was demonstrated by antibody supershift assays. The antibody (p65) considerably shifted the major myotrophin-induced NF- κ B binding complex (Fig. 2 A) and PDTC inhibited this binding activity (unpublished data). To define the possible role of PKC in this activation process, neonatal myocytes were treated with calphostin C before treatment with myotrophin. Formation of the NF- κ B complex was partially inhibited in the presence of calphostin C, as shown in Fig. 2 B. Further characterization of NF- κ B activation was performed by Western blot us-

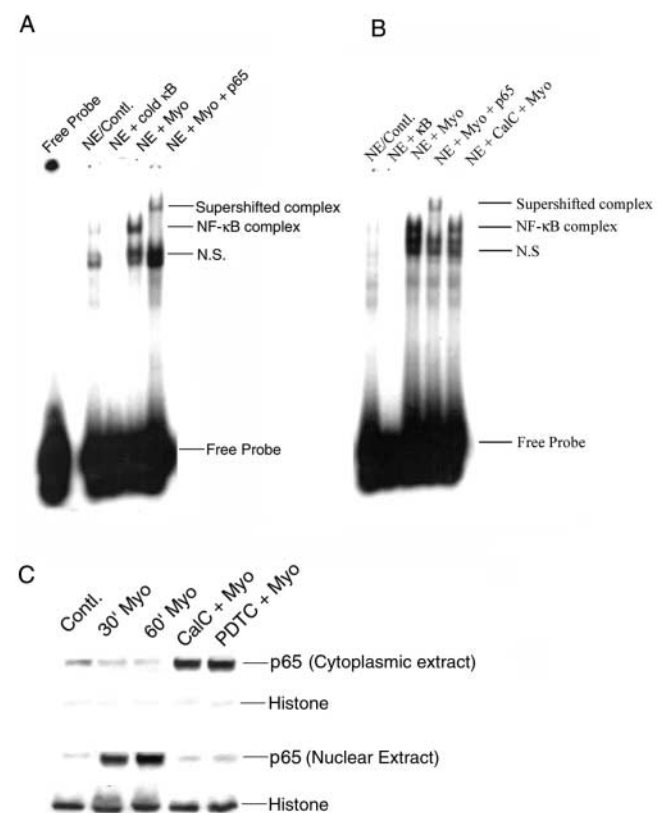
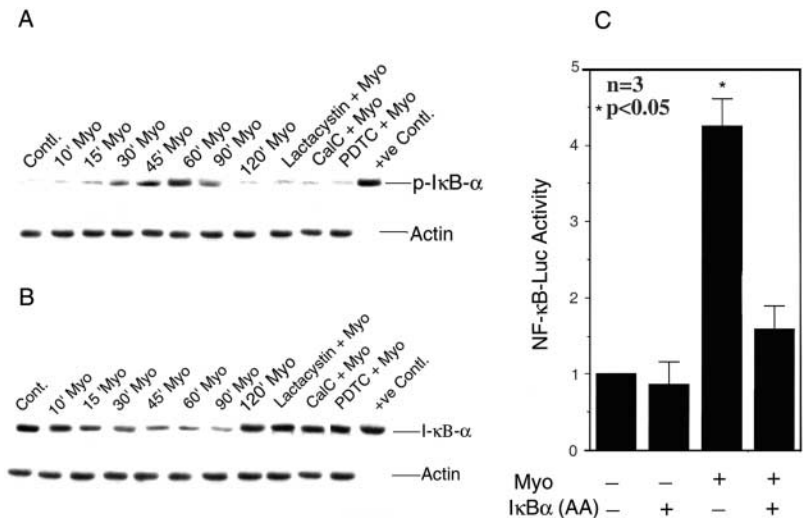


Figure 2. Myotrophin stimulates NF- κ B DNA binding activity. (A) Neonatal myocytes were cultured for 3 d and treated with or without myotrophin for 1 h. Nuclear proteins were isolated and binding reactions were performed with an NF- κ B oligonucleotide labeled with [32 P]dATP. The complex identified could be specifically eliminated with excess unlabeled NF- κ B oligonucleotide. The complex formation was also confirmed by supershift analysis using p65 antibody. This figure is representative of five different experiments. (B) Cells were cultured for 1 h in the presence or absence of calphostin C and then treated with myotrophin for 1 h. The remainder of the procedure was performed as described in A. (C) Western blotting analysis of translocation of NF- κ B p65 protein into the nucleus. Cells were treated with myotrophin for 30 and 60 min. In another set, cells were treated with calphostin C and PDTC before the treatment with myotrophin. Cytoplasmic and nuclear fractions were made and Western blots were performed using a p65 mAb. The bottom panel shows the Western blot using histones as a standard for nuclear proteins.

Figure 3. NF- κ B activation by myotrophin involves phosphorylation and degradation of I κ B α .

(A) Neonatal myocytes were serum starved and incubated in the absence (control) or presence of myotrophin for various time periods. Cells were harvested and cell extracts (50 μ g) were analyzed for phosphorylation of I κ B α using a kit from Cell Signaling Technology. Endogenous actin (bottom) was analyzed as a control. (B) Intracellular levels of I κ B α were also determined in the absence (control) or presence of myotrophin using the same kit as stated above. Endogenous protein levels were determined using an actin antibody (bottom). (C) Neonatal cardiomyocytes were transiently transfected with 2X NF- κ B-Luc (1 μ g/well), with or without the repressor I κ B α (32Ala/36Ala) mutant (200 ng/well). Cells were serum starved and then treated with myotrophin for 24 h or left untreated. Cells were then harvested and Luc activity was determined as described in Fig. 1 B. The results are presented as the mean \pm SEM and represent three individual experiments. $P < 0.05$ compared with the control (untreated).



ing a p65-specific monoclonal antibody. Cardiomyocytes were pretreated with calphostin C and PDTTC before myotrophin. Fig. 2 C shows the Western blot profile of cytoplasmic and nuclear fractions from these cells. Our data indicate that myotrophin induces NF- κ B translocation to the nucleus within 30 min of treatment, whereas calphostin C and PDTTC prevent the translocation, suggesting that PKC is involved in the translocation process. To verify that no contamination of nuclear protein in the cytoplasmic fraction had occurred, we performed Western blots with an antihistone monoclonal antibody. Our data showed that there was no histone protein in the cytoplasmic fraction, suggesting that the fraction was devoid of nuclear protein.

Activation of NF- κ B by myotrophin in neonatal myocytes depends on phosphorylation and degradation of I κ B α proteins and activation of the IKK complex

A key regulatory step in NF- κ B activation is stimulation-induced, ubiquitination-dependent degradation of I κ B α proteins by the 26S proteasome (Traenckner et al., 1994; Thanos and Maniatis, 1995; Whiteside, 1995), a process catalyzed by the IKK complex (Brockman et al., 1995; Thanos and Maniatis, 1995; DiDonato et al., 1997; Regnier et al., 1997; Woronicz et al., 1997; Rothwarf et al., 1998; Yamaoka et al., 1998). However, NF- κ B can also be activated independently of stimulation-induced degradation of I κ B α proteins and IKK activation (Imbert et al., 1996; Li and Karin, 1998; Frost et al., 2000b; Purcell et al., 2001b). To determine the molecular mechanism of NF- κ B activation by myotrophin, neonatal myocytes were treated with myotrophin at various time points (10 min to 2 h) and I κ B α phosphorylation and degradation were analyzed. Treatment with myotrophin induced phosphorylation of I κ B α at 15 min that peaked at 60 min and then began to decrease (Fig. 3 A). Corresponding to the phosphorylation of I κ B α proteins, degradation (Fig. 3 B) began 15 min after treatment with myotrophin, peaked at 60 min, and then recov-

ered at 120 min due to newly synthesized I κ B α , which is one of the target genes of NF- κ B (Brown et al., 1995; Chen et al., 1995; Finco and Baldwin, 1995; Baldwin, 1996; May and Ghosh, 1997; Li et al., 1999). In both cases, the level of actin protein was unchanged (Fig. 3, A and B, bottom). Lactacystin, an inhibitor of the threonine protease of the proteasome, inhibited myotrophin-induced I κ B α phosphorylation and degradation (Fig. 3, A and B). These results suggest that myotrophin-induced degradation of I κ B α proteins is a phosphorylation-dependent process. Furthermore, lactacystin prevented the nuclear translocation of NF- κ B in the myotrophin-treated neonatal myocytes, as evidenced by EMSA (unpublished data). To determine whether PKC was involved in this process, myocytes were treated with calphostin C and both the phosphorylation and degradation statuses of I κ B α were measured. We observed that myotrophin-induced I κ B α phosphorylation and degradation were completely inhibited in the presence of calphostin C, suggesting that PKC may indeed play a role in this process (Fig. 3, A and B). To further determine the molecular mechanism of NF- κ B activation during this initiation process of hypertrophy, neonatal myocytes were cotransfected with the 2X NF- κ B-Luc gene with or without the expression vector encoding the I κ B α (32Ala/36Ala) mutant, which is resistant to stimulation-induced degradation and functions as a suppressor of NF- κ B activation. Cells were treated with myotrophin for 24 h or left untreated. Expression of the I κ B α mutant completely blocked the stimulation of NF- κ B-Luc activity by myotrophin (Fig. 3 C). These data, together, suggest that stimulation-dependent I κ B α degradation is required for myotrophin-induced NF- κ B activation.

The IKK complex mediates activation of NF- κ B by various extracellular stimuli, such as TNF- α and IL-1 (Karin, 1999; Israel, 2000). To determine whether the myotrophin-induced activation of NF- κ B in cardiomyocyte hypertrophy is mediated by IKK β , neonatal cardiomyocytes were transiently transfected with the 2X NF- κ B-Luc reporter gene, with or without expression vectors encoding HA-IKK β (Fig.

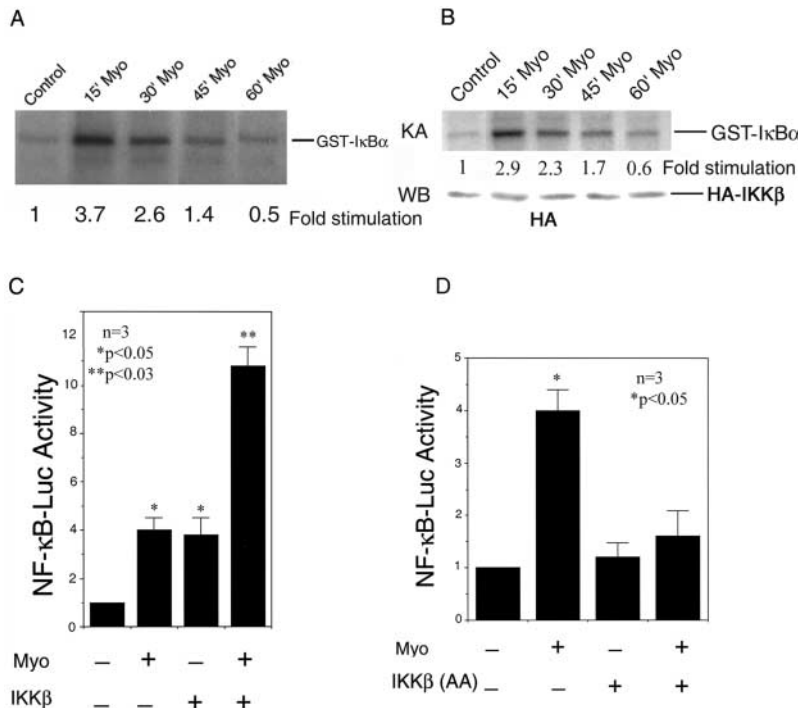


Figure 4. Activation of NF-κB by myotrophin is mediated through the IKK complex. (A) Neonatal myocytes were treated with or without (control) myotrophin for various times. Cells were harvested, cell extracts (500 μg) were immunoprecipitated with IKKβ antibody, and kinase activity was determined by using GST-IκBα as a substrate (Li et al., 2001). (B) Neonatal cardiomyocytes were transfected with HA-IKKβ (1 μg/well) or empty vector. After 36 h, cells were treated with myotrophin (40 nmol) for various times or left untreated, as indicated. HA-IKKβ was immunoprecipitated and immunocomplex kinase assays were performed using GST-IκBα as a substrate as previously described (Li et al., 2001). Substrate phosphorylation was quantitated and the fold induction is given in the bottom panel. An aliquot of each lysate was analyzed for HA-IKKβ content by immunoblotting using HA antibody. (C) Neonatal cardiomyocytes were cotransfected with 2X NF-κB-Luc reporter plasmid (1 μg/well) and expression vector HA-IKKβ (200 ng/well) or (D) the dominant-negative HA-IKKβ (177Ala/181Ala) (50 ng/well) or empty vector, as indicated. Cells were treated with myotrophin for 24 h or left untreated. Cells were washed, harvested, and Luc activity was determined as described in the legend for Fig. 1 B. The results are presented as the mean ± SEM and represent three individual experiments.

4 C), HA-IKKβ (177Ala/181Ala) mutant (Fig. 4 D), or vehicle. Cells were treated with myotrophin for 24 h or left untreated. Fig. 4 C shows the approximately fourfold activation of the NF-κB-Luc reporter gene (lane 2) in myotrophin-treated cells. Expression of wild-type IKKβ potentiated the stimulation of the NF-κB-Luc reporter activity by myotrophin, leading to a 10.8-fold activation (Fig. 4 C, lane 4). Potentiation of the NF-κB-Luc reporter activity by IKKβ and myotrophin was synergistic because IKKβ alone only induced a 3.8-fold activation (Fig. 4 C). We examined whether the HA-IKKβ (177Ala/181Ala) mutant, which has been previously shown to function as a dominant-negative mutant to block NF-κB activation by several stimuli (Nemoto et al., 1998a), was able to block myotrophin-induced NF-κB transcriptional activity. Expression of the dominant-negative IKKβ (177Ala/181Ala) mutant significantly inhibited the NF-κB-Luc activity by myotrophin (Fig. 4 D). PDTC and calphostin C blocked myotrophin-induced NF-κB transcriptional activity (unpublished data). Taken together, these data suggest that myotrophin-induced activation of NF-κB is mediated by the IKKβ complex and PKC is involved.

If the IKK complex mediates the activation of NF-κB by myotrophin in neonatal cardiomyocytes, myotrophin should stimulate IKK activity. Fig. 4 A shows that myotrophin can stimulate IKKβ activity. After 15 min of myotrophin treatment, IKKβ activity was stimulated 3.7-fold (Fig. 4 A). The enhanced activity persisted for up to 30 min and then decreased. Primary cardiomyocytes were also transfected with HA-IKKβ, followed by treatment with myotrophin at various times (15–60 min). We observed that HA-IKKβ was transiently activated 15 min after treatment with myotrophin (a 2.9-fold increase) followed by a gradual decrease (Fig. 4 B). This activation was not a result of increased HA-IKKβ expression, as confirmed by immunoblotting (Fig. 4

B, bottom). The data suggest that myotrophin-induced NF-κB activation is mediated by IKKβ.

Myotrophin enhances IκB-α mRNA expression

Because myotrophin can stimulate NF-κB, we investigated the activation of one of its downstream target genes, IκB-α. To determine the level of IκB-α mRNA expression in myotrophin-treated neonatal myocytes, we performed Northern blot analysis using cDNA for IκB-α and GAPDH as an internal control. Myocytes were treated with myotrophin at various time points (15, 30, 45, and 60 min) and RNA was prepared. The results are shown in Fig. 5. Myotrophin stimulated the expression of IκB-α mRNA in a time-dependent manner. IκB-α mRNA levels increased 3.05- and 3.3-fold after 15 and 30 min of myotrophin treatment, respectively, and peaked (3.9-fold) at 45 min compared with that of con-

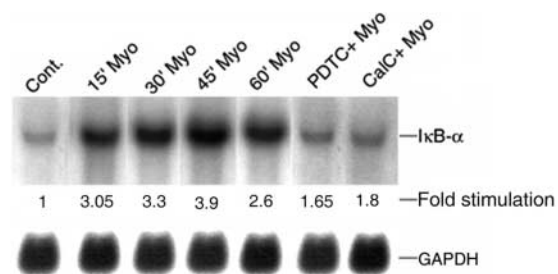
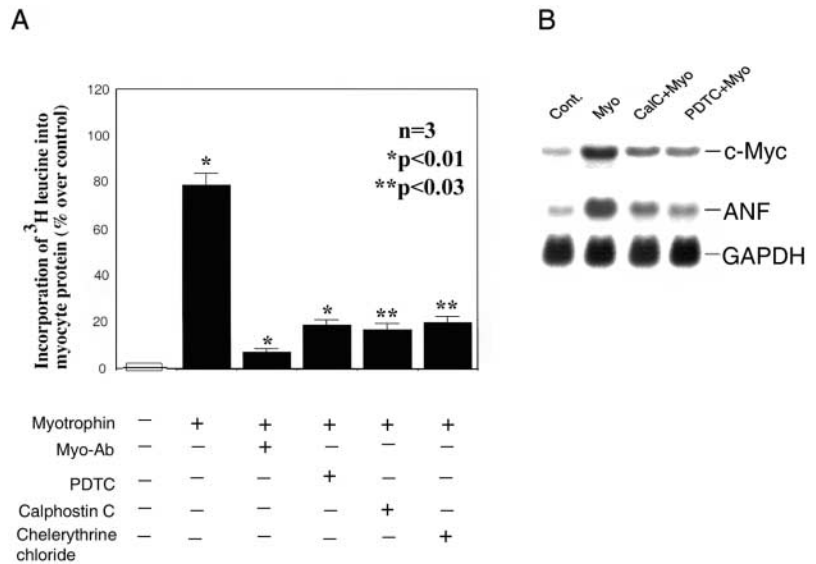


Figure 5. Myotrophin induces expression of IκBα mRNA. Neonatal myocytes were incubated in the absence (control) or presence of myotrophin at various times. RNA was extracted as described in the Material and methods. IκBα mRNA expression was determined using a cDNA probe for IκBα labeled with [³²P]dCTP. Results are presented in three individual experiments. A GAPDH oligo was used as a probe for an internal loading control. Fold stimulation is indicated (lower).

Figure 6. Effect of PDTC, calphostin C, and chelerythrine chloride on myotrophin-induced protein synthesis and gene expression in neonatal rat cardiomyocytes. (A) Neonatal myocytes were pretreated with myotrophin antibody (Myo-Ab), 100 μ M PDTC, 1 μ M of calphostin C, and 10 μ M chelerythrine chloride for 60 min and then treated with myotrophin for 24 h in the presence of [3 H]leucine. Cells were lysed and incorporation of [3 H]leucine into myocytes was measured as described in the text. Data shows the means (\pm SEM) of three different sets of results. P values compared with control or unstimulated cells. (B) Samples from control and myotrophin-treated neonatal myocytes were treated with or without 100 μ M PDTC and 1 μ M of calphostin C. Total RNA (20 μ g) was hybridized separately with [32 P]dATP-labeled *c-myc*, ANF, or GAPDH oligo probes.



control myocytes (no stimulation). PDTC and calphostin C partially inhibited the expression of I κ B- α mRNA (1.65- and 1.8-fold, respectively), suggesting a PKC/NF- κ B-dependent pathway for myotrophin-induced I κ B- α synthesis.

Attenuating NF- κ B inhibits myotrophin-induced protein synthesis and hypertrophic gene expression in myocytes

The experimental results above suggest that NF- κ B may be important for myotrophin-stimulated hypertrophy. To test this scenario, we determined the effect of inhibition of NF- κ B on myotrophin-induced protein synthesis and cardiac gene expression (i.e., ANF and *c-myc*). The effect of blocking NF- κ B by PDTC on myotrophin-induced total protein synthesis in myocytes was analyzed by measuring [3 H]leucine incorporation. Total protein synthesis was increased (\sim 78%) in cells exposed to myotrophin (40 nmol) for 24 h compared with controls ($n = 3$) (Fig. 6 A). Myotrophin-induced protein synthesis was inhibited (\sim 18.6%) by PDTC and also by myotrophin antibody (Myo-Ab) by 6.8% over the control (Fig. 6 A). Myotrophin significantly stimulated PKC activity in neonatal myocytes, and this activity was inhibited by calphostin C (unpublished data). To evaluate the involvement of PKC in myotrophin-induced protein synthesis, calphostin C or chelerythrine chloride were added to the [3 H]leucine incorporation assay. We found that calphostin C and chelerythrine chloride inhibited myotrophin-induced [3 H]leucine incorporation by 16.8% and 18.68%, respectively, over the controls (Fig. 6 A). These results demonstrate that myotrophin-stimulated protein expression may depend on NF- κ B and PKC activity.

An important feature of cardiac hypertrophy is the reexpression of ANF and induction of immediate early genes such as *c-myc* (Lattion et al., 1986; Starksen et al., 1986; Izumo et al., 1988; Mercadier et al., 1989; Gammage and Franklyn, 1991). To determine whether myotrophin-induced expression of different hypertrophic genes, such as ANF and *c-myc*, depends on NF- κ B, we incubated myocytes with or without PDTC and performed Northern blot

analysis. The results are shown in Fig. 6 B. Our data indicated that myotrophin-induced ANF and *c-myc* gene expression was partially inhibited by PDTC. This suggests that NF- κ B may participate in the expression of this hypertrophic marker gene. To determine whether activation of these genes is mediated by PKC, we incubated myocytes with or without calphostin C in the presence of myotrophin. Calphostin C caused inhibition of ANF and *c-myc* expression (Fig. 6 B). Taken together, this suggests that myotrophin-induced hypertrophic gene expression may be PKC and NF- κ B dependent.

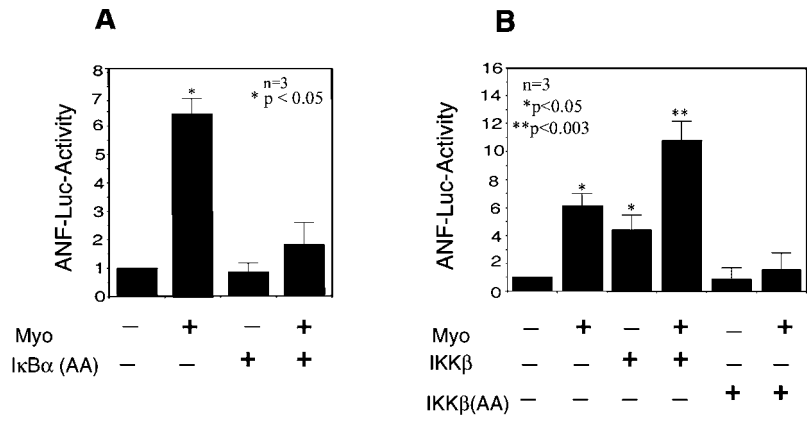
NF- κ B activation may be necessary for the expression of myotrophin-induced ANF in the initiation of cardiac hypertrophy

To evaluate whether NF- κ B is necessary for the transcription of cardiac hypertrophy marker genes in neonatal myocytes, we measured the effect of NF- κ B inhibition on myotrophin-induced expression of ANF. Myotrophin induced ANF-Luc activity 6.1-fold (Fig. 7 A) and expression of the HA-I κ B α (32 Ala/36 Ala) mutant, which inhibits NF- κ B, attenuated the myotrophin-induced ANF-Luc activity (Fig. 7 A). Additionally, myotrophin-induced activation of the ANF-Luc reporter gene was potentiated by expression of wild-type IKK β (10.8-fold) (Fig. 7 B), and the HA-IKK β (177Ala/181Ala) mutant, which is resistant to NF- κ B, attenuated myotrophin-induced ANF-Luc activity (Fig. 7 B).

Discussion

It has been reported that activation of NF- κ B occurs in congestive heart failure and with unstable angina pectoris (Ritchie, 1998; Wong et al., 1998). However, NF- κ B activation in the myotrophin-induced initiation processes of cardiac hypertrophy has not been studied. Our study suggests that NF- κ B activation is necessary for myotrophin-induced hypertrophic gene expression and is PKC dependent. This conclusion is based on several lines of evidence. First, myotrophin stimulated the nuclear translocation of

Figure 7. Inhibition of NF- κ B blocks myotrophin-induced ANF gene expression in neonatal rat cardiomyocytes. (A) Neonatal myocytes were transfected with ANF-Luc reporter plasmid (1 μ g/well) with or without HA-I κ B α (32Ala/36Ala) mutant (200 ng/well), (B) wild-type HA-IKK β (200 ng/well), or the dominant-negative HA-IKK β (177Ala/181Ala) (50 ng/well). Cells were washed and harvested. Luc activity was determined as described in Fig. 1 B. The results are presented as the mean \pm SEM and represent three individual experiments. $P < 0.05$ compared with the control.



NF- κ B and its transcriptional activity in primary cardiomyocytes (Fig. 1, A and B). Also, myotrophin induced NF- κ B DNA binding activity (Fig. 2 A). Second, myotrophin induced phosphorylation and degradation of endogenous I κ B α (Fig. 3, A and B). Lactacystin, a specific proteasome inhibitor, blocked phosphorylation and degradation of I κ B- α by myotrophin (Fig. 3, A and B). Activation of NF- κ B by myotrophin appeared to depend on I κ B α degradation because it was inhibited by expression of the super-repressor I κ B α (32Ala/36Ala) mutant (Fig. 3 C). Furthermore, NF- κ B activation by myotrophin was potentiated by expression of wild-type IKK β (Fig. 4 C) but inhibited by the dominant-negative IKK β (177Ala/181Ala) mutant (Fig. 4 D). Myotrophin consistently stimulated both endogenous IKK β and HA-IKK β activity (Fig. 4, A and B), as well as mRNA expression of an NF- κ B target gene, I κ B α (Fig. 5). Third, myotrophin increased protein synthesis, as quantified by enhanced [3 H]leucine incorporation into myocyte protein, which was inhibited by both PKC and NF- κ B inhibitors (Fig. 6 A). Additionally, we have shown previously that treatment of myocytes with myotrophin resulted in a dose-dependent increase in cell size (quantified by measuring cell surface area) associated with organization of myofibrils, compared with untreated control myocytes (Sen et al., 1990). Myotrophin treatment also enhanced gene expression of ANF and *c-myc* (Fig. 6 B). Fourth, myotrophin-induced gene transcription of ANF was inhibited by expression of the I κ B α (32Ala/36Ala) mutant (Fig. 7 A), or the dominant-negative IKK β (177Ala/181Ala) mutant (Fig. 7 B), but was potentiated by wild-type IKK β (Fig. 7 B). Finally, calphostin C, a PKC inhibitor, attenuated myotrophin-induced NF- κ B activation and expression of ANF and *c-myc* (Figs. 1–3, 5, and 7). This finding suggests that PKC is involved in myotrophin-dependent activation of NF- κ B during the initiation of cardiac hypertrophy in neonatal myocytes.

The kinetics of NF- κ B translocation, I κ B- α degradation, and IKK β activity by hypertrophic agonists such as PE, ET-1, or AngII (Brasier et al., 2000; Rouet-Benzineb et al., 2000; Purcell et al., 2001b) are very similar to those induced by myotrophin presented in this study. In this report, we have shown that myotrophin acts as a potent hypertrophic stimulus that activates NF- κ B through the degradation and phosphorylation of I κ B α in cardiomyocytes to initiate hypertrophy. It is not clear how exogenously added myotro-

phin exerts its action in cultured neonatal myocytes to produce cardiomyocyte hypertrophy. One possibility is that myotrophin has a receptor-mediated mode of action, as evidenced by our previous results (Sil et al., 1998) and those of Anderson et al. (1999), where preliminary studies suggest the presence of a specific myotrophin binding protein in the cardiac membrane. Future work is needed to reveal the details of the mechanism of myotrophin action.

Recently, Knuefermann et al. (2002) reported that myotrophin can interact with NF- κ B (p50 and P65) proteins. The authors have postulated that myotrophin promotes the formation of p50–p50 homodimers from monomeric p50 protein, can convert preformed p50–p65 heterodimers into p50–p50 and p65–p65 homodimers, and appears to play an important role in modulating the level of activated NF- κ B dimers during hypertrophy or heart failure process.

Therefore, data from the present study suggest that myotrophin acts on IKK β (Fig. 4, A–D) and then dissociates the NF- κ B complex (Figs. 2 and 3), thereby allowing NF- κ B to enter the nucleus and activate hypertrophy marker genes (Fig. 6 B). The whole cascade is PKC dependent.

Hypertrophic growth of cardiac myocytes is a complex phenomenon (Thorburn et al., 1994a). Many cellular signaling pathways, e.g., mitogen-activated protein kinases (ERK, JNK, and p38) (Thorburn et al., 1994a,b; Gillespie-Brown et al., 1995; Nemoto et al., 1998b), gp130 (Frost et al., 2000a), and various growth factors (Hefti et al., 1997; Molkentin et al., 1998; Sussman et al., 1998; Olson and Molkentin, 1999), have been reported to be involved in cardiomyocyte hypertrophy. Such in vitro approaches have dissected out some of the pathways involved in the intracellular signal transduction cascade and subsequent activation of a set of genes involved in hypertrophy. Therefore, a lateral crosstalk may occur upon stimulation by a factor(s) that may act alone, or in concert in a synergistic manner (Hefti et al., 1997). Recent studies have shown that the activation of p38 or the ERK pathway may also contribute to NF- κ B activation (Lindroos et al., 1998; Carter et al., 1999; Craig et al., 2000; Vanden Berghe et al., 2000). Therefore, the hypertrophic process is likely an outcome of a combination of many signaling pathways (Chien, 1999, 2000; Vanden Berghe et al., 2000). Further studies are needed to explore the crosstalk between NF- κ B and other signaling pathways to elucidate the mechanisms that initiate cardiac hypertrophy.

Materials and methods

Materials

Timed pregnant rats were obtained from Hilltop Farms. The rats were fed Purina rat chow, given water as needed, and housed in AAALAC-certified facilities. Sprague-Dawley rats weighing 250–300 g (Harlan Sprague Dawley, Inc.) were used for preparation of neonatal myocytes. All experimental procedures used with our animals were in accordance with National Institutes of Health guidelines. The p65 antibody, GST-I κ B α , and double-stranded NF- κ B DNA (5'-AGT TGA GGG GAC TTT CCC AGG C-3') were purchased from Santa Cruz Biotechnology, Inc. IKK β antibody was purchased from Upstate Biotechnology. T4 polynucleotide kinase, [³²P]dATP, [³²P]dCTP, [³H]leucine, protein A-agarose, and NAP-5 column were purchased from Amersham Biosciences. I κ B α degradation and phosphorylation were measured using a kit from Cell Signaling Technology. ANF, *c-myc*, and GAPDH oligonucleotides were purchased from Oncogene Research Products. DME F-12, nonessential amino acids, antibiotics, Tris-borate EDTA, and PBS were purchased from Life Technologies. All other media and reagents used for preparation and culture of the neonatal myocytes, including medium, BSA, insulin, transferrin, fetuin, hydrocortisone, and laminin, were purchased from Sigma-Aldrich. Collagenase was purchased from Worthington Biochemicals. Heparin and pentobarbital were purchased from Elkins-Sinn, Inc. and Abbott Laboratories, respectively. Calphostin C, chelerythrine chloride, lactacystin, PDTC, and protease inhibitor cocktail were purchased from CalBiochem. Alexa[®]488-conjugated goat anti-rabbit IgG and Alexa[®]594-conjugated goat anti-mouse IgG were purchased from Molecular Probes, Inc. Vectashield with DAPI was purchased from Vector Laboratories. All other chemicals used in this study were of molecular biology grade and were purchased from Sigma-Aldrich.

Preparation of neonatal cardiac myocytes

Neonatal myocytes were prepared according to the procedure described by Sen et al. (1990). In brief, hearts from 2–3-d-old normal Wistar Kyoto rat pups were aseptically taken in DME, and the ventricles were separated, minced, and incubated in DME containing collagenase (80 U/ml). Myocytes were plated on laminin-coated wells (20 μ g/35-mm well) at a density of 10⁶ cells/35-mm well. On culture day 3 (or 4), myocytes were incubated in DME alone and were used for the experiment as required. BrdU, at 0.1 mmol/liter, was used throughout the experimental procedure to inhibit the growth of cardiac fibroblast. Myocytes were confirmed by staining with α -actinin (~80–90% myocytes).

Cell treatment

To study the effect of myotrophin on NF- κ B activation, neonatal myocytes were treated with 40 nmol of myotrophin at various times. For inhibitor studies, cells were pretreated with desired inhibitors at specific times. Cells were then washed with cold PBS, harvested, and total cell lysates were prepared. The doses of calphostin C and PDTC were selected after pilot experiments using different doses of the respective inhibitors. A dose of 1 μ M for calphostin C, 10 μ M chelerythrine chloride, and 100 μ M PDTC showed the optimal inhibition without damaging the myocytes.

Measurement of protein synthesis

To determine the total protein synthesis in myotrophin-induced myocyte growth, we performed a bioassay according to Sen et al. (1990). In brief, on culture day 3, neonatal myocytes were preincubated in serum-free DME that contained 1 μ M calphostin C, 10 μ M chelerythrine chloride, or 100 μ M PDTC for 60 min. Then 40 nmol myotrophin was added and the incubation continued for 16–24 h at 37°C. [³H]leucine (10 μ Ci) diluted in DME was added to each well and incubated for 2 h at 37°C. The protein from each individual well was filtered using a cell harvester, collected on individual filter paper discs, and washed extensively with 5% TCA until they were free from radioactivity. The filter papers were air dried and counted in a β scintillation counter after addition of 5 ml scintillation fluid. Data were expressed as disintegration per minute. At least three independent sets of experiments were performed.

Preparation of recombinant myotrophin

Recombinant myotrophin was expressed and purified as described previously (Sivasubramanian et al., 1996).

cDNA constructs and transfection

The following constructs were used in this study and have been described previously (Purcell et al., 2001a): expression vectors encoding HA-tagged IKK β , dominant-negative HA-IKK β (177 Ala/181 Ala) mutant, in which

serines 177 and 181 were replaced by nonphosphorylatable alanine (Ala), and HA-I κ B α (32 Ala/36 Ala) mutant, in which serines 32 and 36 were replaced by Ala. Reporter genes for ANF-Luc and NF- κ B-Luc were described previously by Purcell et al., 2001b. In brief, reporter genes for ANF-Luc, in which the full-length rat promoter (–3003) was fused to the firefly Luc gene, and 2X NF- κ B-Luc, in which two copies of the κ B promoter containing the NF- κ B binding site was fused to the Luc gene.

Transfections and Luc assay

Primary neonatal cardiomyocytes were transiently transfected with the 2X NF- κ B-Luc or ANF-Luc reporter genes with or without various expression vectors encoding HA-IKK β , the dominant-negative mutant HA-IKK β (177Ala/181 Ala), and the dominant-negative HA-I κ B- α (32Ala/36Ala) mutant by using Tfx-20 (Promega) according to the manufacturer's instructions. After transfection, cells were washed four times in plain medium (DME F-12) and maintained in serum-free medium with or without myotrophin, as indicated in the figure legends. Luc activity and normalization of the protein content of each extract were determined by using the dual luciferase reporter assay system (Promega) according to the manufacturer's protocol.

Immunocytochemistry and confocal microscopy

Cell fixation and permeabilization were performed according to the protocol of Rouet-Benzineb et al. (2000). The primary antibody was p65 mAb and the secondary antibody was Alexa[®]568 goat anti-mouse IgG (H+L) conjugate at a 1:1,000 dilution. Immunofluorescent NF- κ B p65 staining was observed using a laser scanning confocal microscope. Confocal images were collected using a Leica TCS-SP spectral laser scanning confocal microscope that was equipped with two argon ion lasers (364 and 488 nm) and a krypton/argon ion laser (568 nm). A PlanApo 20 \times , 0.7 oil immersion objective lens was used. To compare the staining patterns, digitized images for Texas red and DAPI red channels were acquired from the same area and merged to determine overlap in the staining pattern raised from the antibody to NF- κ B p65 and the intercalating agent DAPI.

Cytoplasmic and nuclear protein extracts

After the appropriate experimental treatment, cultures of neonatal myocytes were rinsed with cold PBS. Nuclear and cytoplasmic extracts were made according to the methods described previously by Dignam et al. (1983). All buffers were kept on ice unless stated otherwise. PMSF, DTT, and protease inhibitor cocktail were added just before use. The cytoplasmic and nuclear extracts were normalized for protein amounts determined by Bradford assay using BSA as a standard (Bio-Rad Laboratories protein assay kit). Protein fractions were aliquoted and stored at –70°C.

Western blot analysis

Cytoplasmic and nuclear protein extracts from cardiomyocytes were made as described above. NF- κ B protein was assayed by Western blot in cytoplasmic or nuclear extracts from neonatal myocytes using primary antibody against p65 according to the method described by Rouet-Benzineb et al. (2000). The immunoreactive complex was visualized using chemiluminescence according to manufacturer's protocol (NEN). Phosphorylation and degradation of I κ B α protein was assayed in extracts of neonatal myocytes by Western blotting using a kit from Cell Signaling Technology. Extracts (50 μ g) from each sample were separated by 10% SDS-PAGE. The assays were performed according to the manufacturer's protocol.

Kinase activity assay

Approximately 500 μ g of cytoplasmic proteins from each sample was immunoprecipitated with IKK β antibody and protein A-agarose at 4°C for 1 h. The kinase assay was performed using GST-I κ B α as a substrate according to the protocol by Li et al. (2001).

EMSA

An EMSA was performed using a double-stranded NF- κ B binding site oligonucleotide as a probe. The double-stranded NF- κ B DNA was labeled with [³²P]dATP using T4 polynucleotide kinase and purified using a NAP-5 column. 10 μ g of nuclear protein was incubated in a final volume of 20 μ l binding buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 40 mM NaCl, 5% glycerol, 1 μ g/ml poly [dl:dCl]) on ice for 10 min. The oligonucleotide (50,000 cpm) was added, and the reaction mixture was incubated for an additional 20 min at room temperature. For the supershift assay, specific antibody was added just after ice incubation, and the mixture was incubated for another 10 min at room temperature. For the competition studies, specific competitor (100-fold molar excess) was added along with the binding buffer. After incubation, DNA-protein complexes were sepa-

rated from the unbound DNA probe on a native 4% polyacrylamide gel in low ionic strength buffer (0.25 \times TBE) by electrophoresis at 100 V for 3 h with circulation of the buffer. After electrophoresis, the gel was dried and exposed to Kodak X-AR films at -70°C .

RNA isolation and Northern blot analysis of I κ B α , ANF, and c-myc

Total RNA was isolated from rat neonatal myocytes using the QIAGEN RNA isolation kit. The concentration of total RNA isolated was quantified by UV spectrophotometry at 260/280 nm. For Northern blot hybridization, 20 μg of total RNA was denatured at 70°C for 20 min and fractionated by electrophoresis on 1% agarose gel containing $1\times$ MOPS buffer (20 mM MOPS, 5 mM sodium acetate, and 10 mM EDTA). Northern blotting and hybridization using ANF, c-myc, and GAPDH probes were performed as described previously (Mukherjee et al., 1993). For I κ B α probe preparation, an I κ B α cDNA (provided by Allan Braiser, University of Texas Medical Branch, Galveston, TX) was made using a random primer labeling system (Promega).

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