NF-κB-dependent expression of nitric oxide synthase is required for membrane fusion of chick embryonic myoblasts

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The activity of nitric oxide synthase (NOS) has recently been shown to increase transiently but dramatically in chick embryonic myoblasts that are competent for fusion and that NO acts as a messenger for the cell fusion. Here we show that the rise in NOS activity is tightly correlated with an increase in NOS protein level, and its synthesis is under transcriptional control. In addition, a partial cDNA sequence of NOS obtained by reverse transcription PCR on total RNA from chick myoblasts was found to be identical with that of the inducible type of NOS (iNOS) from chick macrophage. Thus chick myoblast NOS must belong to the family of iNOS. Consistently, pyrrolidine dithiocarbamate, a potent inhibitor of nuclear factor κB (NF-κB), prevented the expression of myoblast NOS. Furthermore the antioxidant also strongly inhibited cell fusion, and its inhibitory effect was reversed by treatment with sodium nitroprusside, an NO-generating agent. In addition, nuclear extracts obtained from myoblasts that were competent for fusion, but not those from proliferating cells or from fully differentiated myotubes, were capable of binding to the consensus NF-κB site in the promoter region of the gene encoding iNOS. These results suggest that NF-κB-dependent expression of NOS is an important step in membrane fusion of chick embryonic myoblasts.

INTRODUCTION

NO is a short-lived free radical which serves as a messenger molecule for diverse physiological or pathophysiological functions [1,2]. It participates in blood vessel relaxation, neurotransmission and antimicrobial activities. In addition, it has recently been demonstrated to act as a mediator of myoblast fusion [3]. The activity of NO synthase (NOS), an enzyme that catalyses the stoichiometric conversion of L-arginine to NO and L-citrulline, increases transiently but dramatically in chick embryonic myoblasts that are competent for membrane fusion. Furthermore treatment with specific inhibitors of NOS, such as N^G-monomethyl-L-arginine, represses myoblast fusion. However, it is not known how the expression of NOS is regulated during the myogenic process.

At least three NOS isoforms have been cloned and characterized in mammals. The brain and endothelial forms of NOS, termed cNOS, are constitutively expressed and regulated by Ca^{2+} and calmodulin in response to neurotransmitters or hormones [1,4]. On the other hand, the inducible isoform (iNOS), found in macrophages and other cells, is known to be induced by cytokines or endotoxins [5]. The activity of iNOS is Ca^{2+}-independent apparently because calmodulin is a tightly bound subunit of the enzyme. Recently, a non-mammalian iNOS cDNA has been cloned from chick macrophages [6]. In addition, the 5′-flanking region of the chick iNOS gene has been shown to contain the binding sites for transcription factors, including nuclear factor κB (NF-κB). Furthermore, using a NF-κB inhibitor, it has been demonstrated that NF-κB is involved in the induction of the chick iNOS gene by endotoxins [6].

NF-κB can be activated in many cell types and regulates a wide variety of genes, such as those involved in immune function and development [7,8]. Moreover, it has been reported that certain cytokines can induce iNOS in skeletal muscle [9]. Therefore the present studies were undertaken to determine whether NF-κB is also involved in the expression of NOS in chick embryonic myoblasts and hence in the regulation of myogenic differentiation of the cells.

EXPERIMENTAL

Materials

Culture media were obtained from Life Technologies. Poly(dl-dC)poly(dl-dC) was from Pharmacia–LKB Biotechnology, and isotopes and enhanced chemiluminescence reagent were from Amersham Corp. Pyrrolidine dithiocarbamate (PDTC) and antibodies were purchased from Sigma Chemical Co. The monoclonal anti-(rabbit skeletal myosin) antibody (clone MY-32) that is specific for myosin heavy chain does not react with non-muscle myosin.

Cell culture

Myoblasts from the breast muscle of 12-day chick embryos were prepared as described previously [10]. The cells were plated at a density of 5×10^5/ml in Eagle's minimal essential medium containing 10% (v/v) horse serum, 10% (v/v) chick embryo extracts and 1% (v/v) antibiotics. One day after cell seeding, the culture medium was changed for the same medium but containing 2% chick embryo extracts. The extent of myoblast fusion was expressed as the number of nuclei in fused cells as a percentage

Abbreviations used: NOS, nitric oxide synthase; cNOS, constitutive nitric oxide synthase; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor κB; PDTC, pyrrolidine dithiocarbamate; RT, reverse transcription; SNP, sodium nitroprusside; GST, glutathione S-transferase

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of the total number of nuclei in ten randomly chosen fields under a microscope. Cells containing more than three nuclei were regarded as fused cells.

Preparation of myoblast extracts

Cultured myoblasts were harvested in ice-cold PBS. They were then sonicated in homogenization buffer (50 mM Hepes, pH 7.4) containing 1 mM EDTA, 1.25 mM $CaCl_2$, 1 mM dithiothreitol, 1 mM PMSF, 10 μM leupeptin, 1.5 μM pepstatin and 10 μg/ml aprotinin. After centrifugation of the samples for 30 min at 15000 ***g*** at 4 °C, the supernatants were passed through 1 ml columns of AG 50W ($\times 8$; Na^+ form) to remove endogenous arginine.

Assays

NOS activity was determined by monitoring the conversion of [^{14}C]arginine into [^{14}C]citrulline as described [11]. Reaction mixtures (0.2 ml) contained 50 mM Hepes (pH 7.4), 0.2 μCi of L-[^{14}C]arginine, 1 mM NADPH, 10 μg/ml calmodulin, 1 mM EDTA, 1.25 mM $CaCl_2$, 1 mM dithiothreitol, 1 mM PMSF, 10 μM leupeptin, 1.5 μM pepstatin, 10 μg/ml aprotinin and appropriate amounts of myoblast extract. After incubation for 10 min at 37 °C, the reaction was terminated by the addition of 0.1 vol. of 20 % (v/v) $HClO_4$. The samples were applied to 1 ml columns of AG 50W ($\times 8$; Na^+ form) and eluted with 2 ml of distilled deionized water. Aliquots of the eluates (i.e. [^{14}C]citrulline) were then counted for radioactivity. DNA synthesis was assayed by measuring the incorporation of [^{3}H]thymidine as described [12]. Proteins were assayed as described by Bradford [13].

Cloning of native and mutant forms of myoblast NOS cDNA

Myoblast NOS primers were synthesized on the basis of the conserved nucleotide sequences in the cDNAs of iNOS from mouse macrophage [14] as well as in those from human endothelial and brain cells [15,16]. The sequences of the primers were 5′-TCTCCATTCCCAAAGGTGCT-3′ (antisense) and 5′-CACCAGGAGATGTT(C/G)AACTA-3′ (sense). To obtain native NOS cDNA, reverse transcription (RT)–PCR was performed using the primers and total RNA obtained from chick myoblasts (see below). The resulting DNA product (361 bp) was inserted into the pBluescript SK vector (Stratagene). To generate a mutant NOS cDNA, the recombinant plasmid was cut by *Nsi*I and then blunt-ended using T4 DNA polymerase. The linearized plasmids were ligated with the 234 bp *Hae*III fragment of $\phi \times 174$ (see Figure 2A). The plasmid containing mutant NOS cDNA was used as the template in *in vitro* transcription to obtain mutant NOS cRNA.

Competitive RT-PCR

Total RNA was isolated from chick myoblasts by acid guanidinium isothiocyanate-phenol-chloroform extraction [17]. Both myoblast total RNA and the mutant form of NOS cRNA were co-reverse-transcribed with avian myeloblastosis virus reverse transcriptase in 20 μl reaction mixtures. The RT reaction was carried out at 42 °C for 1 h. PCR was then performed using the RT products as the DNA templates. PCR amplification was carried out by a two-step procedure for preventing hybrid formation: the first step included denaturation at 94 °C for 1 min, primer annealing at 53 °C for 0.5 min and primer extension at 72 °C for 1 min, and the second step included denaturation of the hybrid at 85 °C for 1 min, primer annealing at 60 °C for 1 min and primer extension at 72 °C for 1 min. To detect the NOS transcripts from cultured myoblasts, 35 cycles of the first step and five cycles of the second were carried out. PCR products were then subjected to electrophoresis on 2 % agarose gels in Tris/borate/EDTA buffer.

Production of anti-NOS antibody

Fusion of the 361 bp fragment of myoblast NOS cDNA with glutathione S-transferase (GST) was generated by PCR-assisted cloning into the pGEX-2T vector (Pharmacia–LKB). The DH5α cells carrying the construct were grown overnight. The cells were diluted 1:100 with Luria broth, cultured to a density of 0.3 A_{600}, and induced with 0.1 mM isopropyl thio-β-galactoside for 2 h. The 43 kDa GST–NOS fusion protein, which is equivalent to the sum of 29 kDa GST and 13 kDa NOS fragment (i.e. 119 amino acids), was then purified from the isopropyl thiogalactoside-treated cells as described [18]. To prepare the antibody against the myoblast NOS, 0.5 mg of the purified fusion protein was electrophoresed on 10 % (w/v) polyacrylamide slab gels containing SDS under reducing conditions [19]. After brief staining of the gels with Coomassie Blue R-250, the 43 kDa bands were cut out, minced and injected into albino rabbits three times at 4-week intervals.

On immunoblot analysis (see below), the antiserum raised against the 43 kDa protein was found to interact specifically with the 43 and 125 kDa protein bands in lysates of *Escherichia coli* and cultured chick myoblasts respectively. The anti-IgG prepared from the antiserum, but not the preimmune IgG, inhibited NOS activity in chick myoblasts in a dose-dependent manner (results not shown).

Immunoblot analysis

Immunoblot analysis was performed as described [20] with minor modifications. Myoblast extracts were prepared as described [21], and aliquots (50 μg) electrophoresed on 8 % mini-gels containing SDS under reducing conditions; the proteins in the gels were then transferred to poly(vinylidine difluoride) membranes. The membranes were incubated with 5 % (w/v) BSA in TBST [100 mM Tris/HCl, pH 8.8, 150 mM NaCl, 0.1 % (v/v) Triton X-100] and then allowed to react with polyclonal antibody raised against the GST-NOS fusion protein (diluted 1:200 with TBST containing 3 % BSA) or with monoclonal antibody against myosin. After three washes with TBST, the membranes were incubated with horseradish peroxidase-conjugated goat anti-IgG (1:4000). Proteins in the membranes were then visualized by treatment with enhanced chemiluminescence reagent as recommended by the manufacturer.

Electrophoretic mobility-shift assay

An oligonucleotide probe containing the decameric consensus NF-κB-binding site and the probe carrying a single mutation were synthesized using an automated DNA synthesizer (Applied Biosystems, model 384A). Mutant probes were prepared by replacing the underlined bases (i.e. G and C) with C and G respectively. The probe was end-labelled with [γ-^{32}P]ATP using T4 kinase.

5′-AGTTGAGGGGACTTTCCCAGGC-3′

3′-TCAACTCCCCTGAAAGGGTCCG-5′

The nuclear extracts of chick myoblasts were prepared as described [22]. Binding reactions (in total 20 μl) were performed

by incubating 10 μg of the nuclear extract with the reaction buffer containing 20 mM Hepes, pH 7.9, 1 mM EDTA, 60 mM KCl, 12 % glycerol and 4 μg of poly(dI-dC)·poly(dI-dC) in the presence or absence of a competitor for 10 min and then with the probes (20000 c.p.m.) for 25 min at room temperature. The reaction mixtures were then subjected to electrophoresis at 30 mA for 2 h on 5 % polyacrylamide gels in high ionic strength buffer (50 mM Tris, 380 mM glycine, 2 mM EDTA, pH 8.5) under non-denaturing conditions [23]. After electrophoresis, the gels were dried and autoradiographed.

RESULTS

Expression of NOS during myogenesis

In order to isolate NOS cDNA from chick embryonic myoblasts, we designed degenerate oligonucleotides corresponding to regions flanking the calmodulin-binding site in mammalian iNOS and cNOS. Using these oligonucleotides as primers, a 361 bp cDNA fragment was obtained by RT–PCR on total RNA from chick myoblasts. Analysis of chick myoblast NOS cDNA sequence showed 100 % identity with the iNOS cDNA sequence in chick macrophage [6] (results not shown). Thus chick myoblast NOS must belong to the iNOS family.

NOS activity in chick embryonic myoblasts has been shown to increase transiently but dramatically just before membrane fusion [3]. To determine whether this increase in NOS activity is due to changes in NOS expression during myogenesis, immunoblot analysis was performed using antibody raised against the GST-fusion protein containing the chick iNOS fragment. The anti-NOS antibody specifically interacted with a 125 kDa protein in extracts of chick embryonic myoblasts (Figure 1). Moreover, the level of this protein markedly increased in fusion-competent cells (i.e. over 36–48 h), became maximal in cells engaged in rapid fusion (48–60 h), and gradually declined thereafter to a level that could no longer be detected (96 h). As a control, accumulation of myosin heavy chain in differentiating myoblasts is also shown (Figure 1). In addition, the anti-NOS antibody, but not the preimmune serum, inhibited the activity of the chick myoblast NOS in a concentration-dependent manner (results not shown). These results indicate that the 125 kDa protein is responsible for the NOS activity in chick embryonic myoblasts and that the changes in NOS activity during myogenesis are due to alterations in the level of the protein.

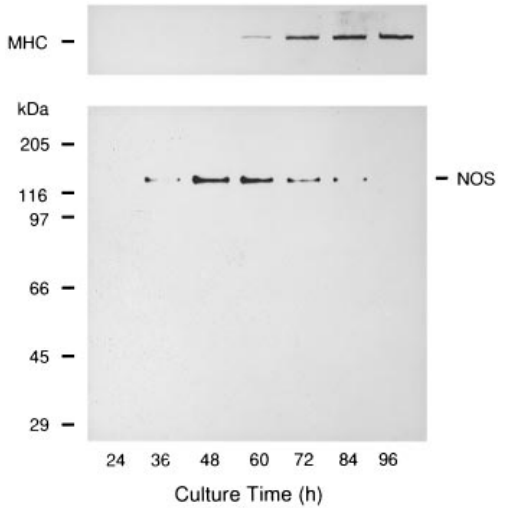

Figure 1 Changes in level of NOS protein during differentiation of chick embryonic myoblasts

Extracts were obtained from the cells cultured for various periods, and aliquots subjected to PAGE under denaturing conditions followed by immunoblot analysis as described in the Experimental section. Expression of myosin heavy chain (MHC) was also examined as above but using a monoclonal antibody, MY-32. The size markers used were myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), BSA (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa).

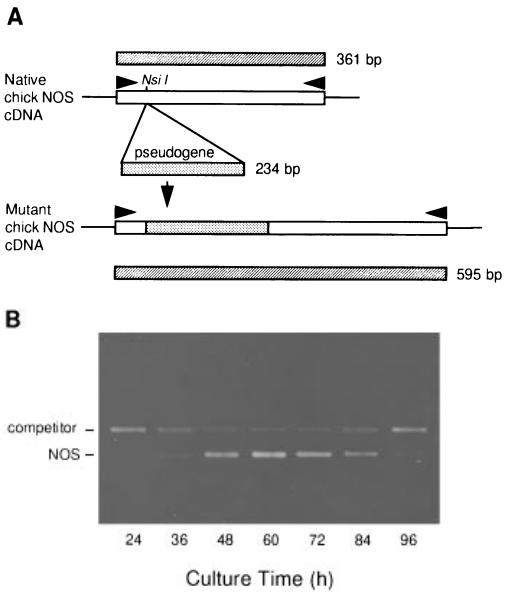

Figure 2 Changes in the level of chick myoblast NOS transcript during myogenesis

(**A**) A 361 bp cDNA fragment for myoblast NOS was cloned into a pBluescript plasmid. The NOS cDNA was also disrupted by a 234 bp fragment of $\phi \times 174$ DNA for generation of its mutant form as described in the Experimental section. (**B**) Competitive RT–PCR was performed using 0.1 pg of the mutant form of NOS cRNA and 1 μg of total RNA obtained from the myoblasts that had been cultured for the indicated period.

To clarify further the temporal expression of NOS during myogenesis, competitive RT–PCR was performed using the mutant form of NOS cRNA as a competitor. As shown in Figure 2(B), the changes in the level of NOS mRNA closely correlated with the alterations in NOS protein level (see Figure 1) as well as with its activity [3]. Thus the expression of NOS is under transcriptional control and must in some way be induced in the cells that are competent for membrane fusion.

Inhibition of NOS expression by PDTC

The promoter of the genes encoding mammalian and chick macrophage iNOS has been shown to contain an NF-κB-binding site, which confers inducibility by bacterial lipopolysaccharide and certain cytokines [10,24]. Therefore it appeared possible that NF-κB may also be involved in the induction of myoblast NOS expression during myogenesis. To test this possibility, we examined the effect of PDTC, a specific inhibitor of NF-κB, on the expression of NOS in chick myoblasts [25]. As shown in Figure 3, PDTC inhibited both the accumulation of NOS and myoblast fusion in a concentration-dependent fashion. Furthermore PDTC at concentrations below 1 μM strongly inhibited the accumulation of NOS mRNA as well as NOS activity (Figure 4, Table 1). Similar data were obtained when the cells were treated with other NF-κB inhibitors, such as butylated hydroxyanisole and

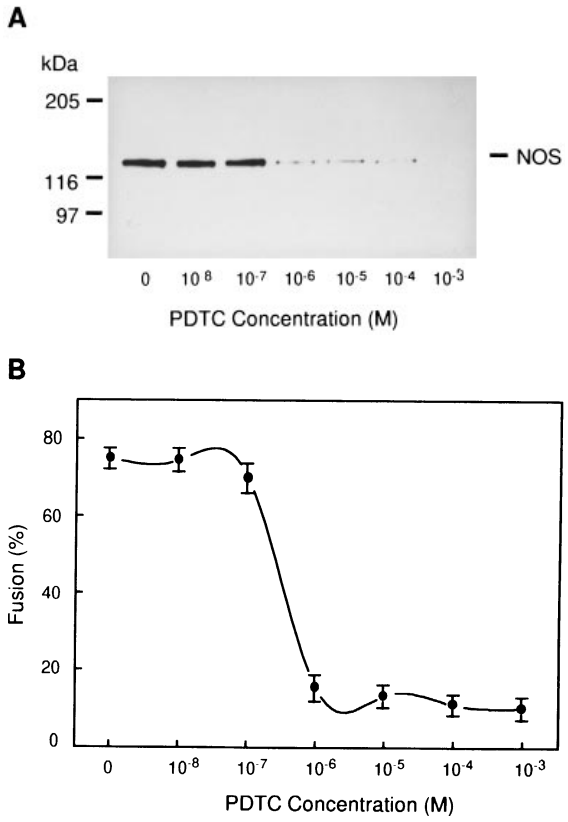

Figure 3 Effects of PDTC on NOS expression and myoblast fusion

The cells that had been cultured for 24 h were treated with increasing amounts of PDTC. (**A**) 24 h after the treatment, the cell extracts were prepared and subjected to immunoblot analysis. (**B**) Another set of PDTC-treated cells was cultured for 48 h and observed under a phase-contrast microscope in order to determine the extent of membrane fusion. Each value represents the mean ± S.E.M. for three independent experiments.

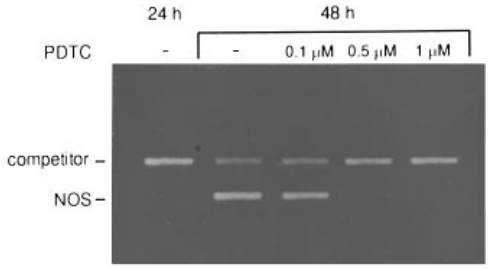

Figure 4 Effects of PDTC on the accumulation of NOS transcript in cultured myoblasts

Myoblasts that had been cultured for 24 h were incubated for 24 h in the presence or absence of various amounts of PDTC. Total RNA was obtained from the cells and co-amplified with 0.1 pg of the mutant form of NOS cRNA. The same experiments were performed with the cells incubated for 24 h in the absence of PDTC as a control.

diethyldithiocarbamate (results not shown). These drugs showed little or no effect on cell proliferation as determined by incorporation of [^{3}H]thymidine (results not shown), indicating that the inhibition of NOS expression is not due to any non-specific cytotoxic effect of the NF-κB inhibitors. These results clearly suggest that NF-κB is involved in the expression of NOS during myogenesis.

Table 1 Effect of PDTC on NOS activity in cultured myoblasts

The cells cultured for 24 h were incubated for 24 h in the presence or absence of various amounts of PDTC. Enzyme activity was determined as described in the Experimental section. The same experiments were performed with the cells incubated for 24 h in the absence of PDTC as a control. Each value represents the mean ± S.E.M. for three independent experiments.

Culture time (h)	PDTC (μM)	$10^{-3} \times$ NOS activity (c.p.m./mg)
24	—	0.72 ± 0.33
48	—	5.92 ± 1.24
48	0.1	5.68 ± 1.67
48	0.5	1.53 ± 0.42
48	1	0.48 ± 0.29

Table 2 Effects of SNP on PDTC-mediated inhibition of myoblast fusion

The cells cultured for 24 h were incubated for 24 h in the presence or absence of PDTC. After the incubation, they were treated with increasing amounts of SNP and further cultured for the next 24 h. The extent of myoblast fusion was determined as described in the Experimental section. Each value represents the mean ± S.E.M. for three independent experiments.

PDTC (μM)	SNP (μM)	Fusion (%)
—	—	76.8 ± 3.3
0.5	—	28.5 ± 5.2
0.5	50	68.3 ± 6.1
0.5	100	72.4 ± 3.5

We have previously shown that sodium nitroprusside (SNP), an NO-producing agent, is capable of not only inducing precocious myoblast fusion but also reversing the fusion-blocking effect of NOS inhibitors, such as N^G-monomethyl-L-arginine [3]. To examine whether the PDTC-mediated inhibition of myoblast fusion is due to prevention of NO generation, the cells that had been cultured in the presence or absence of PDTC were treated with SNP. As shown in Table 2, the SNP treatment effectively reversed the PDTC-mediated inhibition of myoblast fusion. These results indicate that inhibition of NF-κB by PDTC blocks the expression of NOS and consequently prevents the production of NO, which acts as a mediator of myoblast fusion.

Activation of NF-κB during myogenesis

To clarify further the involvement of NF-κB in myogenic differentiation, an electrophoretic mobility-shift assay was performed using radioactive oligonucleotide probes containing the palindromic NF-κB-binding site [26]. The nuclear extracts obtained from myoblasts that were competent for fusion exhibited NF-κB-binding activity (Figure 5, lane b) but not those from rapidly proliferating cells (lane a). In addition, the same non-radioactive probes effectively competed for binding (lane c). On the other hand, the oligonucleotide probe containing a single mutation in the NF-κB-binding site did not show any competition (lane d). Furthermore extracts from cells that had been treated with PDTC showed no NF-κB-binding activity (lane e). These results strongly suggest that NF-κB is in some way activated during the early period of myogenesis for the initiation of myoblast fusion.

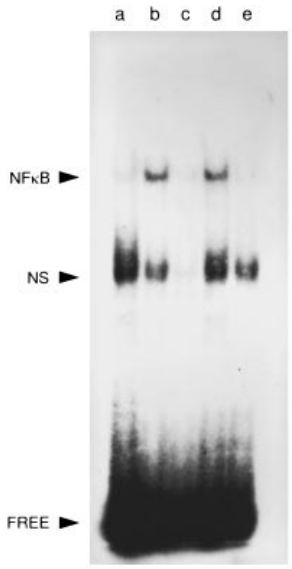

Figure 5 Electrophoretic mobility-shift assay for NF-κB-binding activity

Myoblasts that had been cultured for 24 h (lane a) were further cultured for 24 h in the absence (lane b) or presence (lane e) of 1 μM PDTC. Nuclear extracts were obtained from the cells and subjected to electrophoretic mobility-shift assay using oligonucleotide probes containing the consensus of NF-κB-binding sequence as described in the Experimental section. Nuclear extracts from the cells cultured for 48 h without PDTC were also analysed as above but in the presence of 25-fold molar excess of unlabelled probe (lane c) or the probe carrying a mutation (lane d). NS, non-specific protein–DNA binding.

DISCUSSION

We have previously shown that the activity of NOS in chick skeletal-muscle embryonic myoblasts increases transiently but dramatically just before membrane fusion and that NO generated upon the increase in NOS activity acts as a messenger for the fusion process [3]. In the present studies, we have demonstrated the changes in NOS activity are due to changes in the protein level of the enzyme. Furthermore the alterations in the level of NOS protein are faithfully correlated with that of NOS mRNA on analysis by competitive RT–PCR (see Figures 1 and 2B), indicating that NOS expression in cultured myoblasts is under transcriptional control. This result is consistent with our finding that the partial cDNA sequence of NOS in chick embryonic myoblasts is identical with that of chick macrophage iNOS [6]. Thus it appears that the increase in iNOS expression during myogenesis is responsible for the production of NO in fusion-competent myoblasts.

It has well been documented that the cytokine- and bacterial lipopolysaccharide-dependent induction of iNOS is associated with activation of members of the NF-κB/Rel transcription factor family [27,28]. Several lines of evidence in this report indicate that NF-κB in chick skeletal-muscle myoblasts is activated during myogenic differentiation and its activation is required for cell fusion. PDTC, a specific inhibitor of NF-κB, is capable of blocking not only the expression of NOS but also membrane fusion. In addition, the concentration-dependent inhibitory effect of the agent on the accumulation of NOS mRNA is tightly correlated with the decrease in NOS activity (Figure 4, Table 1). Furthermore the electrophoretic mobility-shift assay shows that NF-κB-binding activity is present in cells that are competent for membrane fusion, but not in proliferating myoblasts or fully differentiated myotubes (Figure 5 and results not shown). Of particular interest is the finding that SNP, an NO-generating agent, can effectively reverse the PDTC-mediated inhibition of myoblast fusion. However, SNP is unable to reverse the inhibitory effect of PDTC on the induction of NOS expression (results not shown). Therefore it is likely that NF-κB is involved in an early step in the signalling pathway that leads to production of NO molecules for the induction of myoblast fusion.

An important but unanswered question involves the timing of NF-κB activation for the induction of NOS expression. None of the NF-κB inducers so far identified, such as endotoxin, interleukin 1β, interferon γ and tumour necrosis factor α, showed any effect on NF-κB activation in cultured chick myoblasts (results not shown). On the other hand, it has recently been demonstrated that cell-shape changes can exert specific effects on gene expression [29]. A variety of agents and conditions that depolymerize microtubules were found to activate NF-κB and induce NF-κB-dependent gene expression in HeLa cells. One of the most prominent events in myogenic differentiation is the conversion of spindle-shaped mononucleated myoblasts into elongated multinucleated myotubes. Since massive cytoskeletal reorganization should be prerequisite for myoblast fusion, it is possible that changes in the state of the cytoskeleton in fusion-competent myoblasts may also act as a signal for NF-κB activation.

In mammalian cells, activation of NF-κB has been shown to require phosphorylation and ubiquitination of IκB followed by degradation of the inhibitor protein by the 26 S proteasome [30]. In addition, we have previously shown that the proteolytic activity of the 20 S proteasome, the catalytic core of the 26 S enzyme, markedly increases in chick skeletal-muscle tissues obtained from day 9–12 embryos [31]. We have also demonstrated that NOS activity dramatically increases in embryonic muscle tissues from embryonic day 10, reaches a maximum by day 13, gradually declines from day 14–15, and disappears almost completely by day 20 [3]. Analysis of the number of nuclei in chick leg and pectoralis muscle tissues has revealed that at embryonic day 9, only about 10 % of all nuclei are in myotubes, but by day 18, about 80 % are in myotubes [32]. Therefore it is possible that an increase in 20 S proteasome activity and hence in activity of the 26 S enzyme complex may also occur in cultured myoblasts that are competent from membrane fusion and that the increased activity may be responsible for IκB degradation and thus for nuclear translocation of NF-κB/Rel transcription factor. We have recently found that lactacystin, a specific inhibitor of the 20 S proteasome, can block myoblast fusion (K. H. Lee and M. S. Kang, unpublished work). The effects of proteasome inhibitors as well as of microtubule-disrupting agents on the expression of myoblast NOS are at present under investigation.

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