Inhibitors of the proteasome block the myogenic differentiation of rat L6 myoblasts

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Abstract Myogenesis is characterized by membrane fusion and accumulation of muscle specific proteins. We have previously shown that nitric oxide acts as a messenger for membrane fusion. Here we show that inhibitors of the proteasome, such as lactacystin, reversibly block both the fusion of L6 myoblasts and the accumulation of muscle specific proteins, such as myosin heavy chain (MHC). The inhibitors also reversibly prevented the induction of the NF-κB activity, which is required for the expression of nitric oxide synthase (NOS). Moreover, the inhibition of the NF-κB activity occurred in parallel with that of the NOS activity upon treatment with increasing concentrations of lactacystin. While pyrrolidine dithiocarbamate, an inhibitor of NF-κB, blocked both membrane fusion and accumulation of MHC, N^G-monomethyl-L-arginine, a specific inhibitor of NOS, inhibited only the fusion. These results suggest that the proteasome plays an essential role in the regulation of myogenic differentiation through the activation of NF-κB and that the target of NF-κB for the expression of muscle specific proteins is distinct from that for myoblast fusion.

Key words: Proteasome; Lactacystin; Nuclear factor κB; Myogenesis; Nitric oxide synthase

1. Introduction

Myogenesis, a well-defined differentiation event, is characterized by fusion of mononucleated myoblasts into multinucleated myotubes [1,2]. Concurrent with the morphological differentiation, a large number of muscle specific proteins, such as α-actin and myosin, accumulate during the myogenic process [3,4]. Protein breakdown appears to play an important role in regulation of muscle cell differentiation [5]. For example, calpain and its specific inhibitor protein, called calpastatin, have been implicated in the control of myoblast fusion [6–9].

The 26S proteasome that is the ATP-dependent protease in eukaryotes consists of two multimeric components: the catalytic 20S core and the regulatory 19S ATPase [10]. This enzyme complex degrades target proteins that are conjugated with ubiquitin, such as cyclins and transcription factors (e.g. Jun, Fos, and NF-κB/IκB) [11]. Thus, the ubiquitin-dependent protein breakdown catalyzed by the 26S proteasome plays a critical function in regulation of a variety of cellular processes, including cell cycle regulation, gene expression, cell differentiation, and immune response. In addition, we have shown that the subunit pattern and the poly-L-lysine activated casein degrading activity of the 20S proteasome core dramatically changes during development of chick embryonic muscle [12].

NF-κB is a transcription factor that can be activated in many cell types and regulates a wide variety of genes involved in immune function and development [13,14]. For example, it positively regulates the expression of the inducible form of nitric oxide synthase (NOS), which catalyzes the conversion of L-arginine to NO and L-citrulline [15,16]. NO is a short-lived free radical which serves as a messenger for diverse physiological functions, such as smooth muscle relaxation, neurotransmission, and antibacterial activity [17,18]. In the absence of inducer, NF-κB is trapped in cytoplasm by an inhibitor protein known as IκB. In the presence of inducer, however, IκB is phosphorylated, ubiquitinated, and degraded by the 26S proteasome, to release NF-κB, which can then be translocated into the nucleus for expression of NOS as well as others [19,20]. Thus, the 26S proteasome is essential for activation of NF-κB.

We have previously demonstrated that NO acts as a messenger for myoblast fusion [21] and that the NF-κB dependent expression of NOS is required for membrane fusion of chick embryonic myoblasts [22]. In the present study, we examined whether the 26S proteasome plays an essential role in NF-κB mediated regulation of myogenic differentiation, using specific inhibitors of the 26S proteasome.

2. Materials and methods

2.1. Cell culture

The L6 rat myogenic cell line [23] was obtained from American Type Culture Collection. The cells were plated on plastic tissue culture dishes at a concentration of 1.5×10^4 cells/ml in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum [24]. Three days after cell seeding, the culture medium was changed to a differentiation medium, which contained 5% horse serum instead of 10% fetal bovine serum. When needed, proteasome inhibitors were treated to the culture medium at the time of medium change. The time of the medium change was regarded as day 0 of culture (see below). The extent of myoblast fusion was expressed as the number of nuclei in fused cells as a percentage of the total number of nuclei in 10 randomly chosen fields under a microscope. Cells containing more than three nuclei were regarded as fused cells.

2.2. Electrophoretic mobility shift assay

An oligonucleotide probe containing the decameric consensus NF-κB binding site sequence [22,25] was synthesized using an automated DNA synthesizer (Applied Biosystem, model 384A). The probe was end-labeled with [γ-^{32}P]ATP using T4 kinase. The nuclear extracts of L6 cells were prepared as described [26]. Binding reactions (in 20 μl total) were performed by incubating 10 μg of the nuclear extract with a reaction buffer containing 20 mM HEPES (pH 7.9), 1 mM EDTA, 60 mM KCl, 12% (v/v) glycerol, and 4 μg poly(dI-dC)·poly(dI-dC) for

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10 min at 4°C and then with the probe (about 80 000 cpm) for the next 25 min at the same temperature. The reaction mixtures were electrophoresed on 5% (w/v) polyacrylamide gels in high ionic strength buffer containing 50 mM Tris (pH 8.5), 380 mM glycine, and 2 mM EDTA [27]. The gels were then dried and autoradiographed.

2.3. Assays

The activity of NOS was determined by monitoring the conversion of [^{14}C]arginine (Amersham) into [^{14}C]citrulline as described [28]. 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 of calmodulin, 1.25 mM $CaCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 1.5 μM pepstatin, 10 μg/ml of aprotinin, and appropriate amounts of myoblast extracts. After incubation for 10 min at 37°C, the reaction was terminated by adding 20 μl of 20% (v/v) $HClO_4$. The samples were applied to 1 ml columns of AG 50W (×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.

3. Results and discussion

3.1. Effects of proteasome inhibitors on myogenic differentiation

In order to determine whether the proteasome is involved in differentiation of L6 myoblasts, the cells were cultured in the presence and absence of 1 μM lactacystin, 100 nM *N*-carbobenzoxy-Leu-Leu-norvalinal (MG115), or 10 nM *N*-carbobenzoxy-Ile-Glu-(*O*-*t*-butyl)-Ala-leucinal (PSI), which specifically inhibit the proteasome [29–31]. As shown in Fig. 1A, these reagents strongly inhibited myoblast fusion, although this inhibitory effect was slowly diminished upon prolonged culture (e.g. at day 3). At the concentrations tested, they showed little or no cytotoxic effect, as assessed by the rate of [^{3}H]thymidine incorporation (data not shown). We then examined whether the inhibitory effect on myoblast fusion can be reversed upon removal of the reagents. The cells cultured for 24 h in the presence of 1 μM lactacystin were freed of the inhibitor by changing the medium, and further cultured for the next 48 h. Fig. 1B shows that myoblast fusion can be resumed upon removal of the inhibitor. Similar data were obtained when

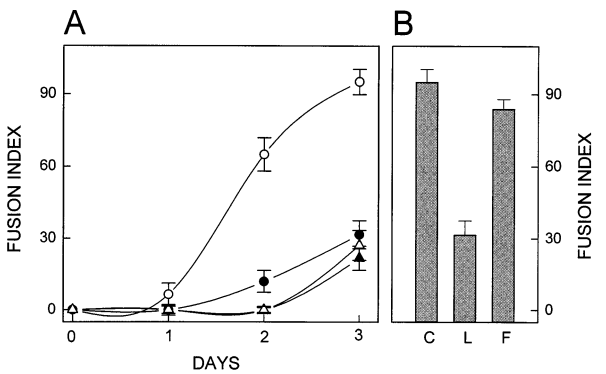

Fig. 1. Effects of the proteasome inhibitors on membrane fusion of L6 myoblasts. A: The cells were plated and cultured for 72 h as described in Section 2. After the culture (i.e. at day 0), the culture medium was changed to differentiation medium. The cells were then further cultured for various periods in the absence (○) and presence of 1 μM lactacystin (●), 10 nM PSI (▲), and 100 nM MG115 (△). The extent of myoblast fusion was then determined. B: The cells that had been incubated in the absence (lane C) and presence of 1 μM lactacystin (lane L) for 24 h were freed of the reagent (lane F) by changing to fresh differentiation medium and further cultured for the next 48 h.

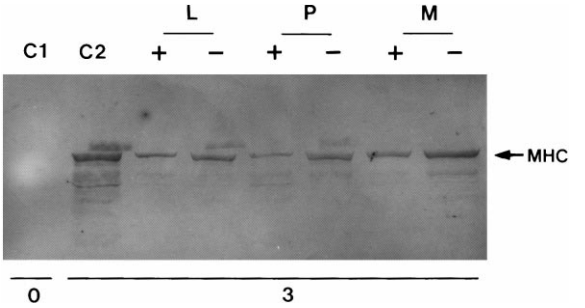

Fig. 2. Effects of the proteasome inhibitors on accumulation of myosin heavy chain. After the medium change, the cells were cultured in the absence of the inhibitors for 24 h (lane C1) or 72 h (lane C2). They were also cultured in the presence of the inhibitors ('+' lanes) for 72 h. To determine whether their effects are reversible, the cells treated with the inhibitors for 24 h were freed of the reagents by changing the medium and further cultured for the next 48 h ('−' lanes). The cell lysates were then prepared, electrophoresed in 10% polyacrylamide slab gels under denaturing conditions, and subjected to immunoblot analysis using a monoclonal antibody (MF-20) raised against myosin heavy chain (MHC). The numbers at the bottom of the gel indicate the days after cell plating. L, lactacystin (1 μM); P, PSI (10 nM); M, MG115 (100 nM).

the cells were treated with MG115 and PSI, following removal of the inhibitors (data not shown). These results indicate that the proteasome inhibitors block myoblast fusion and their effects are reversible.

To examine whether the proteasome inhibitors also influence accumulation of muscle specific proteins during myogenesis, L6 myoblasts were cultured in the presence and absence of lactacystin, MG115, or PSI as above. After the culture, the cell lysates were prepared and subjected to immunoblot analysis using the anti-myosin heavy chain (MHC) monoclonal antibody (MF-20). Fig. 2 shows that the accumulation of MHC is significantly reduced upon the treatment ('+' lanes) but can be resumed upon removal of the inhibitors ('−' lanes). These results indicate that the proteasome inhibitors block not only the membrane fusion but also the synthesis of muscle specific proteins and that both of their effects are reversible. Thus, it seems that the proteasome plays a critical role in regulation of myogenic differentiation.

3.2. Effects of proteasome inhibitors on the activation of NF-κB

Proteasomes are known to degrade ubiquitinated IκB for activation of NF-κB [19,20]. In addition, we have previously shown that NO acts as a messenger for myoblast fusion and that NF-κB dependent expression of NOS is essential for membrane fusion of chick embryonic myoblasts [21,22]. Therefore, we first examined the expression pattern of NF-κB in nuclear extracts of L6 myoblasts during the time course of myogenic differentiation by probing it with a radiolabeled oligonucleotide containing the decameric consensus NF-κB binding site. As shown in Fig. 3A, the NF-κB activity was dramatically increased 24 h after the change of the culture medium to differentiation medium (i.e. at day 1) and rapidly disappeared 48 h after the medium change (i.e. from day 2). Furthermore, the transient increase in the NF-κB activity occurred just prior to the onset of myoblast fusion (see Fig. 1A) as well as of accumulation of muscle specific proteins (see below). Thus, the timely increase in the NF-κB activity appears to be critical for myogenic differentiation.

On the other hand, when the cells were treated with lacta-

cystin at the time of medium change, little or no increase in the NF-κB activity was observed at day 1 (Fig. 3B). These results indicate that the proteasome activity, most likely against IκB, is responsible for induction of the NF-κB activity during myogenic differentiation. Upon prolonged culture of the cells in the presence of the inhibitor, however, the NF-κB activity gradually increased although to a level much less than that seen without the inhibitor. This slight increase in the level of NF-κB activity might be due to slow metabolism of lactacystin in the cells, and is in accordance with the finding that the cells resume myoblast fusion during the late period of culture although to low extents (see Fig. 1A, day 3).

We then examined whether the other proteasome inhibitors can also block the induction of the NF-κB activity and whether the inhibitory effects can be reversed upon removal of the reagents. The cells were cultured for 24 h in the presence of the inhibitors, freed of the inhibitor by changing the medium, and further cultured for the next 24 h. As shown in Fig. 4, the binding of NF-κB to the oligonucleotide probe was significantly reduced upon treatment of the inhibitors ('+' lanes) but could be restored upon their removal ('−' lanes). These results again show that the NF-κB activity is mediated by the action of the proteasome, which is most likely to be the degradation of ubiquitinated IκB.

In order to determine whether the NF-κB activity that increases just prior to myoblast fusion is also related to the expression of genes for muscle specific proteins, the cells were cultured for various periods in the absence and presence of 1 μM pyrrolidine dithiocarbamate (PDTC), an inhibitor of NF-κB [32]. After the culture, the cell lysates were prepared and subjected to immunoblot analysis as above using the anti-MHC monoclonal antibody. Table 1 shows that the treatment of PDTC strongly diminishes the accumulation of MHC. In addition, the same treatment prevented both NF-κB activation and membrane fusion in the cells cultured for 24 h and 72 h, respectively (data not shown). Thus, it appears that the synthesis of muscle specific proteins also requires NF-κB activity.

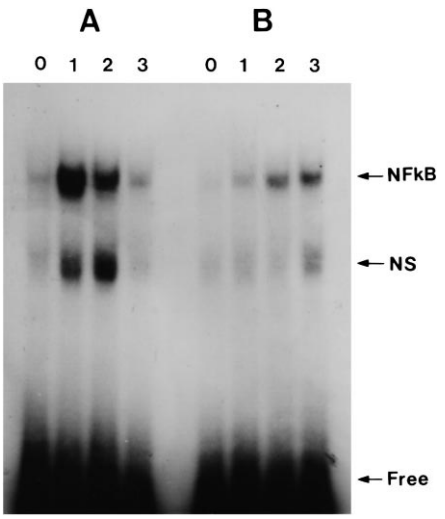

Fig. 3. Effect of lactacystin on the induction of NF-κB activity during myogenesis. The cells were cultured for various periods as in Fig. 1 but in the absence (A) and presence of 1 μM lactacystin (B). Nuclear extracts were prepared from the cells at the indicated culture periods and subjected to electrophoretic mobility shift assay for their NF-κB binding activity using the radiolabeled palindromic NF-κB binding site as a probe as described in Section 2. The numbers at the top of the gel show the day after the medium change. NF-κB, NF-κB/DNA complex; NS, nonspecific; Free, unbound probe. The nucleotide sequence of the probe is: 5′-AGTTGAGGGGACTTTCCCAGGC-3′, 3′-TCAACTCCCCTGAAAGGGTCCG-5′. Note that the fast-migrating bands (indicated as NS), but not the NF-κB binding activity, disappear when the same assays were performed in the presence of an unlabeled, mutant probe, in which the underlined bases (G and C) are replaced by C and G, respectively (data not shown).

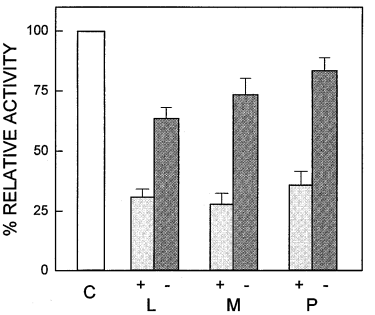

Fig. 4. Effects of the proteasome inhibitors on NF-κB activity. After the medium change, the cells were cultured in the presence of the inhibitors ('+' lanes) for 24 h. To determine whether their effects are reversible, the cells that had been treated with the inhibitors for 24 h were freed of the reagents by changing the medium and further cultured for the next 24 h ('−' lanes). As a control, the cells were also cultured for 24 h in the absence of any inhibitor. Nuclear extracts were prepared from the cells and subjected to electrophoretic mobility shift assay as in Fig. 3. The bands corresponding to the complex of NF-κB and the radioactive probe were scanned using a phosphoimager (Fuji), and the intensity of the band seen without any inhibitor (lane C) was regarded as 100% and the others are values relative to it. L, lactacystin (1 μM); P, PSI (10 nM); M, MG115 (100 nM).

3.3. *Effect of lactacystin on the induction of NOS activity*

We have recently shown that both the activity and protein level of NOS also transiently and markedly increase just before fusion and its expression requires NF-κB activity in cultured chick myoblasts [21,22]. A similar change in NOS activity was observed with L6 myoblasts (data not shown). Therefore, we examined whether the proteasome inhibitors can also block the induction of NOS. L6 myoblasts were treated with increasing concentrations of lactacystin at the time of medium change. Twenty-four hours after the treatment, soluble and nuclear extracts were prepared from the cells and assayed for the activities of NOS and NF-κB, respectively. Upon the treatment, the NOS activity was inhib-

Table 1
Effect of PDTC on the accumulation of MHC

Culture time	Relative amount of MHC in the cells cultured (%)	
	without PDTC	with PDTC
Day 1	14 ± 3	3 ± 1
Day 2	65 ± 1	17 ± 4
Day 3	100	21 ± 3

The cells were cultured in the absence and presence of 1 μM PDTC. At the indicated times of culture, the cell lysates were prepared and subjected to immunoblot analysis as in Fig. 2. The bands corresponding to MHC were scanned using a densitometer (Pharmacia), and the intensity of the band seen with the cells cultured for 72 h without PDTC was regarded as 100% and the others are values relative to that.

Table 2
Effects of increasing concentrations of lactacystin on the induction of NF-κB and NOS and on myoblast fusion

Concentration (μM)	Relative activity (%) of		Membrane fusion (%)
	NF-κB	NOS	
None	100	100	100
0.01	72 ± 2	75 ± 4	83 ± 5
0.1	48 ± 9	51 ± 8	57 ± 2
1.0	30 ± 7	27 ± 3	36 ± 4

L6 myoblasts were cultured as in Fig. 1 but in the absence and presence of increasing concentrations of lactacystin. Soluble and nuclear extracts were prepared from the cells cultured for 24 h and assayed for their activities of NF-κB and NOS, respectively. The cells were also continuously cultured for 72 h for determination of fusion. The activities of NF-κB and NOS and the cell fusion determined in the absence of lactacystin were expressed as 100% and the others are values relative to it. Each value represents the mean ± S.E.M. of data obtained from three separate experiments.

ited in a dose dependent fashion and its inhibition occurred in parallel with the reduction in NF-κB activity (Table 2). When the cells were further incubated for the next 48 h under the same culture condition, membrane fusion was also inhibited in a dose dependent fashion. These results indicate that proteasome is involved in the signaling pathway for the NO-mediated membrane fusion of L6 myoblasts through activation of NF-κB, which leads to expression of NOS.

We have previously shown that the casein degrading activity of the 20S proteasome, which is activated by poly-L-lysine, markedly increases in chick skeletal muscle tissues obtained from 11–17 day embryos [12], at which period the fused myotubes accumulate most actively [33]. We have also demonstrated that the subunit pattern of the 20S proteasome dramatically changes during development of the embryonic muscle [12]. Thus, it is possible that an increase in the 20S proteasome activity, perhaps due to the change in its subunit pattern, and hence in the activity of the 26S proteasome may also occur in cultured L6 myoblasts that are competent for membrane fusion and that the increased activity may be responsible for IκB degradation and hence for activation of NF-κB. The free form of NF-κB is then translocated into the nucleus, where it induces NOS for generation of NO, a messenger for myoblast fusion.

However, NOS does not seem to be involved in the expression of genes for muscle specific proteins. We have previously shown that N^G-monomethyl-L-arginine, a specific inhibitor of NOS, inhibits membrane fusion but shows little or no effect on the accumulation of creatine kinase and acetylcholine receptor in chick embryonic myoblasts [21]. Similar results were obtained when the same reagent was treated to L6 myoblasts (data not shown). Thus, it appears that the target of NF-κB for the expression of muscle specific proteins is distinct from that for myoblast fusion, although both myogenic processes require the activity of proteasome in the activation of NF-κB. More study is required for understanding the molecular mechanism underlying how NF-κB influences the expression of the muscle specific proteins.

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