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p38 mitogen-activated protein kinase up-regulates NF-κB transcriptional activation through RelA phosphorylation during stretch-induced myogenesis

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ABSTRACT

p38 MAPK and nuclear factor-B (NF-B) signaling pathways play an indispensable role in the control of skeletal myogenesis. The specific contribution of these signaling pathways to the response of myoblast to the mechanical stimulation and the molecular mechanisms underlying this response remain unresolved. Using an established in vitro model, we now show that p38 MAP kinase activity regulates the transcriptional activation of NF-κB in response to mechanical stimulation of myoblasts. Furthermore, SB203580 blocked stretch-induced NF-κB activation during myogenesis, not through down-regulation of degradation of IκB-α, and consequent translocation of the p65 subunit of NF-κB to the nucleus. It is likely that stretch-induced NF-κB activation by phosphorylation of p65 NF-κB. Moreover, depletion of p38α using siRNA significantly reduces stretch-induced phosphorylation of RelA and NF-κB activity. These results provides the first evidence of a cross-talk between p38 MAPK and NF-κB signaling pathways during stretch-induced myogenesis, with phosphorylation of RelA being one of the effectors of this promyogenic mechanism. The α isoform of p38MAP kinase regulates the transcriptional activation of NF-κB following stimulation with cyclic stretch.

Myogenesis is a highly orchestrated multistep process in which muscle precursor cells or myoblasts cease to proliferate, and then differentiate and fuse into multinucleated myotubes [1–3]. It is essential for muscle growth and maintenance, as well as for the repair of injured muscle fibers [4]. Accumulating evidences suggest that mechanical stimuli are important regulators in postnatal myogenesis and adaptive response of skeletal muscle [5]. However, the precise mechanisms in which the mechanical stimuli are transduced into intracellular signals that cause changes in the tissue morphology are poorly understood.

Recently, a number of in vitro studies have shown that p38 MAPK and nuclear factor-κB (NF-κB) pathways have been implicated in the control of myogenic progression [6]. Activation of p38 MAPK is an early and essential event in myogenic differentiation in embryos and myoblasts [7–12]. p38 MAPK activates myogenesis through phosphorylating and increasing the transcriptional activity of specific MEF2 isoforms [13], or through stimulating MyoD activity [9]. Contrary to this, NF-κB was shown to induce cyclin D1 expression and pRb hyperphosphorylation during proliferation of myoblasts, thus inhibiting their differentiation [14]. Alternative studies, however, showed that IGF-II induced a transient activation of NF-κB in rat myoblasts, with activation required for IGF-II-mediated differentiation [15]. Thus, the role of NF-κB during myoblast differentiation deserves further analysis.

NF-κB, is a family of transcription factors composed of members of the Rel family [16,17], the activation of which is controlled by the inhibitor of NF-κB (I-κBα) which retains NF-κB in the cytoplasm. The most predominately characterized NF-κB complex is a p50/p65 heterodimer, which is associated with an inhibitor protein, IκB, at rest and is retained in the cytoplasm [18]. Phosphorylation of IκB-α and the p65 subunit of NF-κB is an important event in translocation and in the transcriptional activity of NF-κB [19].

It has been shown that mechanical stimulation activates p38 in muscle [20,21] and in myoblasts that have undergone 2 days of differentiation [22]. Accumulating evidence indicates that once activated p38 MAPK appears to be capable of further signal transduction through kinase phosphorylation; it is also capable of modulating the phosphorylation of transcription factors, including activating transcription factor-2 [23], myocyte enhancer factor 2C [24], C/EBP homologous protein 1 [25], and NF-κB [26]. A recent study has suggested that NF-κB activation was found to be

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dependent on p38 MAPK activity during myoblast differentiation, being an effector of p38 MAPK, thus providing a novel mechanism for the promyogenic effect of p38 MAPK [27]. However, the role of p38 MAPK in the upstream pathway leading to NF-κB activation remains controversial [27–29]. Furthermore, the role of p38 MAPK in NF-κB activation during myogenic differentiation of C2C12 cells provoked by mechanical stimulation is still unknown. Here, we have investigated the role of p38 MAPK in stretch-induced NF-κB activity during myogenic process in cyclically stretched skeletal myoblasts.

Materials and methods

Reagents. Antibodies specific for the phosphorylated forms of p38 MAPK (#9211), p65-Ser276 (#3037), as well as for the total p38 MAPK (#9212) were obtained from Cell Signalling Technology (Beverly, MA 01915, USA). Antibodies specific for the total p65 (#sc-109) and IκBα (#sc-371) were from Santa Cruz Biotechnology Inc. (CA 95060, USA). The antibody against actin (#A2103) was from Sigma–Aldrich Chemie GmbH. Prestained molecular mass markers (#P7708) were from New England Biolabs (Beverly, MA 01915, USA). The selective inhibitors SB203580 (#559389), PD98059 (#513000) were obtained from Calbiochem–Novabiochem (La Jolla, CA, USA).

Cell culture and application of cyclic stretch. The myoblast cell line C2C12 was obtained from American Type Culture Collection and maintained in DMEM supplemented with 10% fetal calf serum (GM) with 5% CO_2 at 37 °C. For myogenic differentiation, cells were switched to fresh DMEM supplemented with 2% horse serum (differentiation medium; DM). Cells were exposed to cyclic stretch as previously described [30,31]. Cells, when switched to DM, were subjected to cyclic stretch at 3 s of 10% stretch alternating with 3 s of relaxation at various times before harvested for differentiation assay and expression of muscle specific differentiation.

Western blotting. Whole cell lysates, cytosolic and nuclear extracts were prepared as described previously [28]. Lysates normalized to protein levels were separated by 10% SDS–PAGE, transferred to nitrocellulose, and detected by standard techniques. Protein concentrations were determined using the BioRad Bradford assay.

Transient transfection and reporter-gene assay. Transient transfection was prepared as described previously [27]. Briefly, C2C12 myoblasts were transiently transfected with LipofectAMINE (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. After transfection either with luciferase reporters (p6xNF-B-Luc), with or without expression plasmids, or only with expression plasmids. Cells were then harvested to measure luciferase activity after exposed to cyclic stretch at various times in the absence or presence of SB203580, PDTC. All transfections included constant amounts of β-galactosidase reporter plasmid, as control for transfection efficiency. The total amount of DNA for each transfection was kept constant using an empty expression vector. Luciferase activities were normalized by dividing luciferase activity by β-galactosidase activity.

p38α RNA interference by siRNA. siRNA against p38α (Cat. No. SC-29434) and a control siRNA (Cat. No. SC-37007) were purchased from Santa Cruz. Cells were grown to 50% confluence in antibiotic-free medium and specific or control siRNA was transfected at the indicated concentrations using LipofectAMINE™ transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were incubated at 37 °C for 48 h to allow maximal knock-down of target genes and then treated with the indicated time of cyclic stretch. Western blotting was performed as indicated.

Statistical analyses. Data are expressed as means ± SEM for at least $n = 3$ independent experiments. Analysis of variance (ANOVA) or Student's t-test was used to compare data. A p value of $p < 0.05$ was considered statistically significant. Statistical analysis was performed using a commercial software package (SPSS Version 11, Chicago, IL).

Results

p38MAP kinase mediates the activation of NF-κB during myogenic differentiation in C2C12 cells stimulated by cyclic stretch

Previous studies indicated that both p38 and NF-κB activities are induced in stretch-induced differentiation and are required for completion of the myogenic program [27]. However, the existence of a potential cross-talk between both pathways during skeletal myogenesis has never been investigated. Thus, we set out to analyze whether NF-κB might function downstream of the p38 MAPK pathway during stretch-induced myogenesis. The activation of NF-κB in C2C12 cells after continuously stretched for 30 min was analyzed by NF-κB-dependent/luciferase reporter assays in the presence of SB203580 (10 μM). As shown in Fig. 1A, NF-κB-dependent luciferase activity was induced after continuously stretched for 30 min, the presence of the inhibitor caused a reduction in the luciferase activity (Fig. 1A), suggesting that p38 MAP kinase is required for NF-κB transcriptional activation following stimulation with cyclic stretch. Similar results were obtained when C2C12 cells that overexpressed a dominant negative form of p38 MAP kinase were stimulated with cyclic stretch. C2C12 cells were cotransfected with plasmids containing the luciferase gene under three repeats of NF-κB consensus binding sequences and dnp38 MAP kinase. Forty hours later, the cells were stretched for 30 min. Overexpression of the dominant negative form of p38 MAP kinase resulted in decreased luciferase activity (Fig. 2B). Taken together, these data suggested that p38 MAP kinase is a necessary component of the signaling pathway leading to the transcription activation of NF-κB target genes in C2C12 cells following stimulation with cyclic stretch.

Cyclic stretch stimulate the activation of NF-κB by p38 MAP kinase-mediated phosphorylation of RelA

In order to investigate a possible mechanism underlying the action of p38 MAPK on NF-κB activation, we first sought to deter-

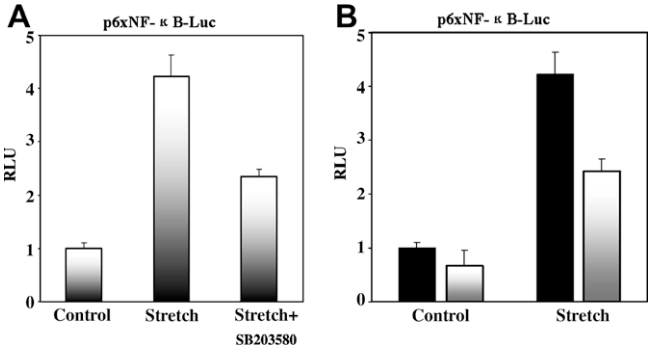

Fig. 1. p38 MAP kinase-dependent NF-κB activity in C2C12 cells stimulated with cyclic stretch. (A) C2C12 cells (3 × 106 cells) after transiently transfected with a luciferase reporter gene construct (p6xNF-κB-Luc) were stimulated with cyclic stretch in the presence of the specific p38 MAP kinase inhibitor SB203580. Luciferase activity was measured after continuously stretched for 30 min. (B) C2C12 (3 × 106 cells) were cotransfected with plasmids encoding the luciferase gene under three repeats of an NF-κB consensus binding sequence as well as a dominant negative form of p38 MAP kinase. After 48 h, the cells were stimulated with cyclic stretch, and luciferase activity was measured as described above. The results presented are representative of three independent experiments.

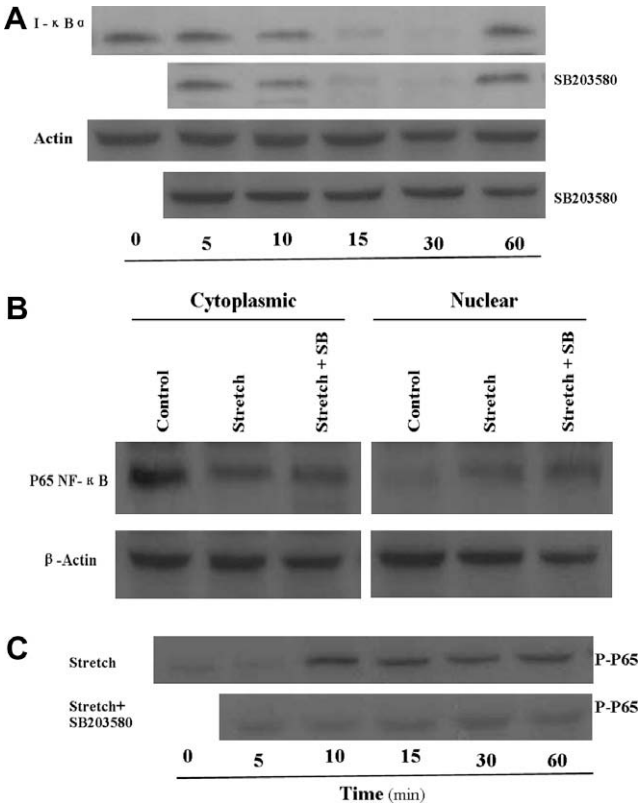

Fig. 2. Degradation of I-κB and nuclear translocation of p65 NF-κB to the nucleus are unaffected by the inhibition of p38 MAP kinase. C2C12 cells (3×10^6 cells) were stimulated with cyclic stretch in the presence or absence of SB203580 (5 μM) for 30 min. Total cell extracts were analyzed for I-κB (A), and p65 NF-κB (B) by Western blotting. Densitometry of bands is expressed in arbitrary densitometric units. (C) p38 MAP kinase signaling mediates the phosphorylation of RelA. C2C12 cells (3×10^6 cells) were stimulated with cyclic stretch in the presence or absence of SB203580 (10 μM) for 30 min. RelA was immunoprecipitated with an anti-RelA antibody and immunoblotted with an anti-phospho-Ser276 RelA antibody. The results presented are representative of three to five experiments.

mine whether p38 MAP kinase inhibition during stimulation of cyclic stretch affected the degradation of I-κBα, the inhibitory subunit of the NF-κB complex. As shown in Fig. 2A, the inhibition of p38 MAP kinase did not prevent the degradation of I-κBα in response to cyclic stretch. Because I-κBα degradation results in the translocation of p65 NF-κB to the nucleus, the cytoplasmic and nuclear pools of p65 NF-κB were also examined by Western blot analysis. Cyclic stretch decreased the amount of p65 NF-κB in the cytoplasm and increased its appearance in the nucleus (Fig. 2B). SB203580 had no effect on the nuclear translocation of p65 to the nucleus. These data suggest that p38 MAPK regulates the stretch-induced pathway that leads to NF-κB activation not through degradation of IκB-α, and consequent translocation of p65 NF-κB to the nucleus.

Some reports previously suggested that p38 MAP kinase is involved in the phosphorylation of RelA, the transcriptionally active subunit of the NF-κB complex at Ser276, leading to the full activation of the transcription factor. Thus, we tested whether the inhibition of p38 MAP kinase activity during cyclic stretch affected the phosphorylation of RelA at this particular residue. C2C12 cells were stretched for 30 min as described above, and nuclear extracts were immunoblotted with an anti-phospho-Ser276 RelA antibody. The inhibition of p38 MAP kinase resulted in reduced RelA phosphorylation (Fig. 2C), indicating that the contribution of p38 MAP kinase-mediated signaling to the activation of NF-κB occurred through the phosphorylation of the transcriptionally active subunit of the NF-κB complex. These data suggest that activation of p38 MAP kinase is a necessary precursor to RelA phosphorylation and transcription of NF-κB target genes.

p38α MAPK mediated stretch-induced activation of NF-κB through phosphorylation of RelA

The p38α is the major isoform involved in muscle differentiation [32]. Therefore, we tested the contribution of the α isoform of p38 MAP kinase to the phosphorylation of RelA. C2C12 cells (3×10^6 cells) were transfected with an siRNA mixture targeting p38 MAP kinase. The transfection resulted in the efficient inhibition of p38α MAP kinase expression, as demonstrated by RT-PCR (Fig. 3A). The siRNA-transfected (and control transfected) cells were then continuously stretched for 30 min, and the phosphorylation status of RelA at Ser276 was determined as described above. The silencing of the p38α MAP kinase gene resulted in diminished RelA phosphorylation after continuously stretched for 30 min compared to siRNA-transfected controls (Fig. 3B). Collectively, these results indicate that p38α MAP kinase signaling is required for the transcriptional activation of NF-κB through the phosphorylation of RelA. Moreover, our results suggest that p38α MAP kinase is the specific isoform involved in the signaling pathway leading to the phosphorylation of RelA in response to cyclic stretch.

Discussion

Numerous investigations indicated that mechanical strain influences muscle physiology at the level of mononucleated satellite cells, which drives cell proliferation and differentiation [22,33,34]. However, the mechanisms implicated in this response are not well defined and, thus, the investigation of strain-induced intracellular signaling regulating the cellular function during cyclic stretch is very important. In the current study, our results demonstrate a central role for p38MAP kinase activity in regulating the activation of NF-κB during stretch-induced myogenesis. We identify that p38 MAP kinase signaling is required for the transcriptional activation of NF-κB through the phosphorylation of RelA. Moreover, our results suggest that p38α MAP kinase is the specific isoform involved in the signaling pathway leading to the phosphorylation of RelA in response to cyclic stretch.

p38 MAPK plays an indispensable role in the activation of the myogenic program [5,11,12]. It has been demonstrated to be activated in response to environmental stresses, including mechanical stretch, and inflammatory cytokines [35,36]. Activation of p38

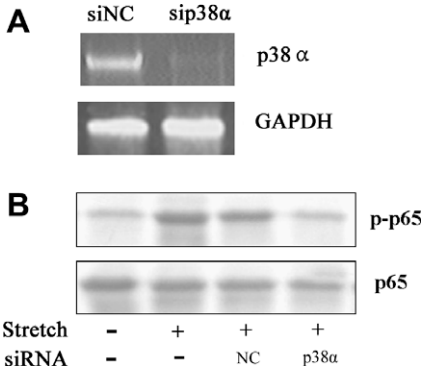

Fig. 3. Specific silencing of the α isoform of p38 MAP kinase results in reduced RelA phosphorylation. (A) C2C12 cells were transfected with siRNA targeting p38α MAP kinase (sip38α). Twenty-four hours after transfection, the cells were analyzed by RT-PCR to determine the efficiency of the transfection. siNC, negative control (NC) siRNA. (B) The cells were stretched continuously for 30 min. Phospho-RelA was determined by Western blotting.

MAPK is an early and essential event in myogenic differentiation in embryos and myoblasts [11,12]. p38 MAPK activates myogenesis through multiple actions on some sequential steps critical to myogenic differentiation [37,38].

NF-κB is a critical transcription factor for maximal expression of many cytokines that are involved in the control of myogenic progression [39]. The p38 and ERK MAPK pathways may play an important role in NF-κB activation, depending on the stimulus [38]. Therefore, it is important to clarify the key regulatory steps that lead to NF-κB activation during stretch-induced myogenesis. Recent studies have suggested that p38 MAPK is involved in NF-κB transcriptional activity dependently of IKK, through serine phosphorylation of the p65 NF-κB subunit in skeletal muscle after physical exercise [39]. Furthermore, p38 inhibition significantly augmente I-κBa degradation and NF-κB DNA-binding activity besides potentiating the transactivating activity of p65 in C2C12 cells [27]. Moreover, p38 MAPK has been proposed to regulate NF-κB indirectly by phosphorylating nucleosome components such as histone H3, an essential step that leads to selective transcriptional activation of NF-κB-dependent gene expression in response to inflammatory stimuli [40,41]. In the present study, our results show that the p38 MAPK inhibitor SB203580 significantly decrease stretch-induced NF-κB-dependent promoter–reporter activity. However, SB203580 do not inhibit nuclear translocation of the NF-κB subunit p65, as shown in Fig. 2. Similarly, treatment with the p38 MAP kinase inhibitor SB203580 has no apparent effect on cyclic stretch-induced IκBα breakdown. However, stretch-induced serine phosphorylation of p65 NF-κB is inhibited by SB203580. Together, these data indicate that stretch-induced p38 MAPK activation modulates NF-κB-dependent transcription, at least in part, through mechanisms independent of NF-κB nuclear translocation, presumably by stimulating the transactivation of the p65 subunit.

In conclusion, data from the present study suggest that cyclic stretch activate two distinct but converging signaling pathways which result in the transcriptional activation of NF-κB during stretch-induced myogenesis. The p38 MAP kinase-independent signaling pathway leads to the translocation and binding of the NF-κB complex to its target DNA, while the p38 MAP kinase-dependent pathway results in the phosphorylation of RelA and thus the activation of NF-κB. Moreover, our results also suggest that the α isoform of p38 MAP kinase regulates the transcriptional activation of NF-κB during stretch-induced myogenesis.

Acknowledgments

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