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Mitochondria Mediate Tumor Necrosis Factor-α/NF-κB Signaling in Skeletal Muscle Myotubes

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ABSTRACT

Tumor necrosis factor-α (TNF-α) is implicated in muscle atrophy and weakness associated with a variety of chronic diseases. Recently, we reported that TNF-α directly induces muscle protein degradation in differentiated skeletal muscle myotubes, where it rapidly activates nuclear factor κB (NF-κB). We also have found that protein loss induced by TNF-α is NF-κB dependent. In the present study, we analyzed the signaling pathway by which TNF-α activates NF-κB in myotubes differentiated from C2C12 and rat primary myoblasts. We found that activation of NF-κB by TNF-α was blocked by rotenone or amytal, inhibitors of complex I of the mitochondrial respiratory chain. On the other hand, antimycin A, an inhibitor of complex III, enhanced TNF-α activation of NK-κB. These results suggest a key role of mitochondria-derived reactive oxygen species (ROS) in mediating NF-κB activation in muscle. In addition, we found that TNF-α stimulated protein kinase C (PKC) activity. However, other signal transduction mediators including ceramide, Ca^{2+}, phospholipase A2 (PLA_2), and nitric oxide (NO) do not appear to be involved in the activation of NF-κB. Antiox. Redox Signal. 1, 97–104.

INTRODUCTION

ACCELERATED MUSCLE PROTEIN DEGRADATION and negative nitrogen balance are the hallmarks of skeletal muscle wasting associated with a variety of chronic diseases that include sepsis, cancer, congestive heart failure, and acquired immunodeficiency syndrome (AIDS) (Epstein, 1996). In each of these diseases, tumor necrosis factor-α (TNF-α) has been implicated as a primary trigger of muscle wasting (Espat *et al.*, 1994). TNF-α is a cytokine involved in immune and inflammatory responses, and its production can be drastically elevated in disease processes, attaining serum levels as high as several ng/ml (Vreugdenhil *et al.*, 1992; Nakashima *et al.*, 1995). Elevated TNF-α levels in laboratory animals result in muscle loss, particularly loss of muscle structural proteins such as myosin heavy chains (Ahmad *et al.*, 1994; Buck and Chojkier, 1996). Recently, we reported that TNF-α directly induces protein loss in differentiated skeletal muscle myotubes (Li *et al.*, 1998). Utilizing this cell culture model of TNF-α-induced muscle catabolism, we have investigated the underlying cellular mechanism. We found that TNF-α rapidly activates transcription factor NF-κB in myotubes (Li *et al.*, 1998) and that TNF-α-induced protein loss is NF-κB-dependent (unpublished observations). Given the importance of TNF-α/NF-κB signaling in muscle, the present study was conducted to assess postreceptor mechanisms that mediate NF-κB activation.

Studies in nonmuscle cells have identified multiple signaling pathways that can mediate

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TNF-α activation of NF-κB. The importance of these pathways varies in a cell-type-dependent manner. For example, reactive oxygen species (ROS) mediate TNF-α/NF-κB signaling in human breast cell lines (Kretz-Remy *et al.*, 1996) and T-cell lines (Schreck *et al.*, 1991; Sappey *et al.*, 1994; Schmidt *et al.*, 1995; Makropoulos *et al.*, 1996), but not in monocytic cell lines (Israel *et al.*, 1992) or epidermal cells (Brennan and O'Neill, 1995). Thus, it is necessary to establish the mechanism of TNF-α signaling in individual cell types of interest.

Recent data indicate that endogenous ROS mediate TNF-α/NF-κB signaling in skeletal muscle cells. In undifferentiated myoblasts from the L6 cell line, NF-κB activation is sensitive to cellular glutathione (Sen *et al.*, 1997). In differentiated myotubes, we demonstrated that exogenous H_2O_2 activates NF-κB and that catalase partially inhibits the activation of NF-κB by TNF-α (Li *et al.*, 1998). However, NF-κB activation by H_2O_2 was slower and less complete than activation by TNF-α. Thus, it was not clear whether ROS are essential for NF-κB activation or are merely a modulating influence.

The present study was designed to assess the importance of mitochondria-derived ROS in NF-κB activation by TNF-α in muscle. We also examined the involvement of other factors that mediate TNF-α/ROS/NF-κB interactions in nonmuscle cell types. Our results indicate that ROS generated by mitochondrial electron transport are essential for NF-κB activation by TNF-α. TNF-α also stimulates protein kinase C (PKC) activity. In contrast, NF-κB activation does not appear to involve ceramide, Ca^{2+}, phospholipase A_2 (PLA_2), or nitric oxide (NO).

MATERIALS AND METHODS

Reagents

Recombinant mouse TNF-α was purchased from Boehringer Mannheim (Indianapolis, IN). Rotenone, amytal, antimycin A, phosphatidylserine, *sn*-1,2-dioctanoylglycerol, C_2-ceramide, C_6-ceramide, SMase, oleoylethanolamine (NOE), ruthenium red, caffeine, nifedipine, melittin, manoalide, and aristolochic acid were obtained from Sigma (St. Louis, MO).

Myogenic cell culture

Myotubes differentiated from either C2C12 or rat (primary culture) myoblasts were used in all experiments. Cell culture methods were described previously (Li *et al.*, 1998). Differentiation was achieved by switching 85% confluent myoblasts to low-serum medium containing 2% heat-inactivated horse serum. The incubation then was continued for 96 hr.

NF-κB activity

NF-κB was activated by treating myotubes with mouse recombinant TNF-α 3 ng/ml for 30 min. Binding of NF-κB to its targeted DNA sequence was measured by electrophoretic mobility shift assay (EMSA) using nuclear extracts as described previously (Li *et al.*, 1998).

PKC assay

PKC activity was determined as described by Klann *et al.* (1998). Briefly, myotubes were scraped off plates and were lysed by brief sonication in a buffer containing 20 m*M* Tris-HCl pH 7.5, 1 m*M* EDTA, 0.5 m*M* EGTA, 2 m*M* sodium pyrophosphate, 100 μM phenylmethylsulfonyl fluoride (PMSF), 25 μg/ml leupeptin, and 25 μg/ml aprotinin. Each sample was incubated in a reaction mixture that contained 100 μM [γ-^{32}P] ATP (5 μCi), 10 m*M* $MgCl_2$, and 10 μM neurogranin$_{28-43}$, a selective PKC substrate (Chen *et al.*, 1993). To assay for basal PKC activity, 2.5 m*M* EGTA was added to the reaction. Stimulated PKC activity was assessed by including 100 μM $CaCl_2$, 320 μg/ml phosphatidylserine, and 30 μg/ml *sn*-1,2-dioctanoylglycerol. Reactions were incubated for 10 min at 37°C and terminated by spotting onto P-81 phosphocellulose filter papers. Radioactivity on the filter papers was measured by scintillation counting after they were washed with 150 m*M* H_3PO_4 and dried.

Western blot analysis

Protein samples were separated using SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were

incubated in the presence of PKC antibody (1:1,000), a gift from Dr A. Newton (Sweatt *et al.*, 1998). A horseradish peroxidase-conjugated second antibody (1:30,000; ICN, Costa Mesa, CA) was used to locate the primary antibody. Antibodies were detected by the en-

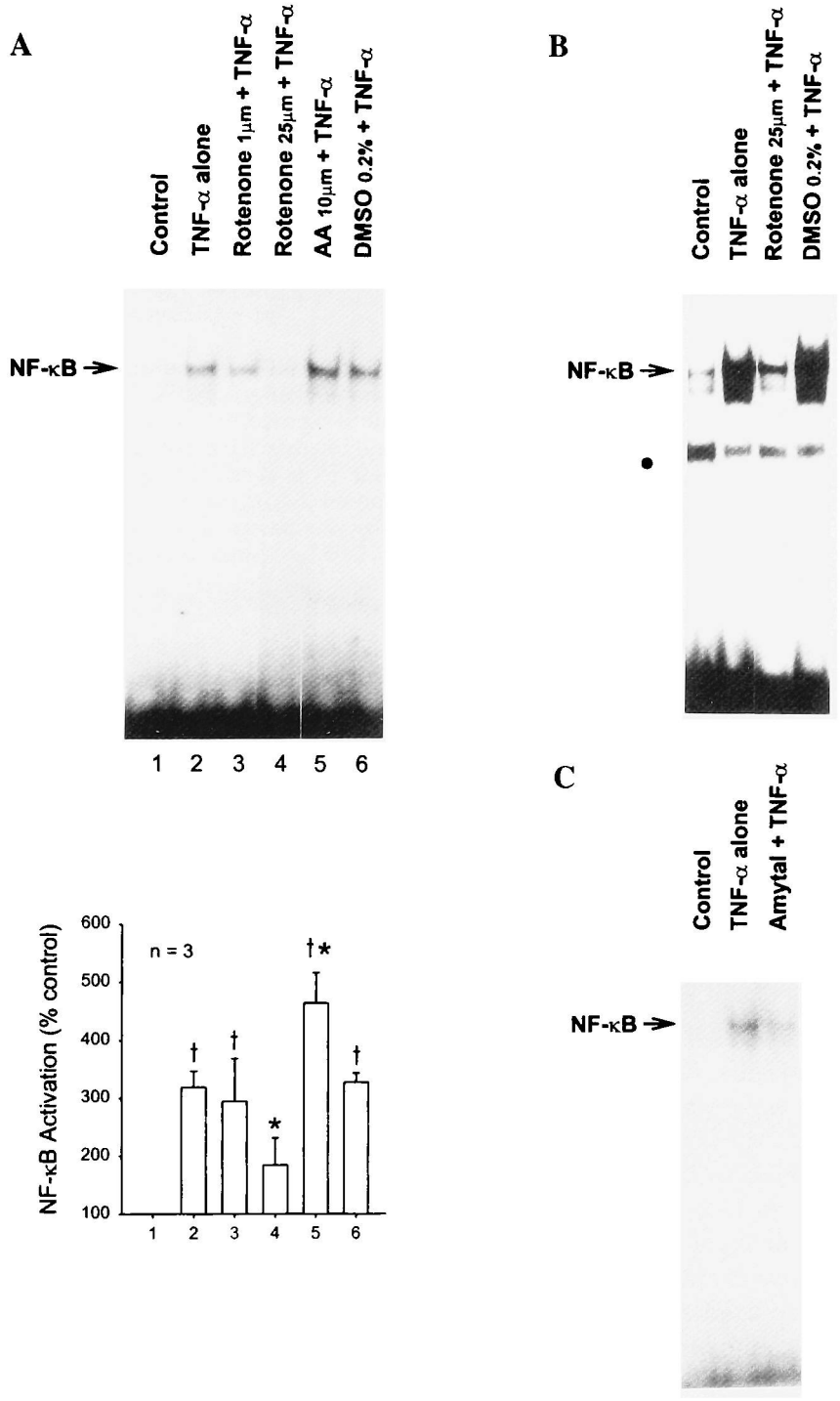

FIG. 1. Effects of mitochondrial inhibitors on NF-κB activation induced by TNF-α. Myotubes were pretreated for 30 min with mitochondrial inhibitors or dimethyl sulfoxide (DMSO, vehicle for reagents) followed by the addition of 3 ng/ml TNF-α for 30 min. NF-κB activation was analyzed by EMSA. Dot indicates nonspecific binding. **A.** Effects of rotenone and antimycin A (AA) on NF-κB activation induced by TNF-α in C2C12 myotubes. Statistical analysis of the data from three experiments using ANOVA indicated an overall difference ($p < 0.05$) among treatments. † indicates a difference from Control determined by a paired *t*-test ($p < 0.05$); * difference from TNF-α alone ($p < 0.05$). **B.** Effects of rotenone on NF-κB activation in rat skeletal muscle myotubes (primary culture). **C.** Effects of 10 μ*M* amytal on NF-κB activation in C2C12 myotubes.

hanced chemiluminescence method (Amersham).

RESULTS

ROS generated by the mitochondrial respiratory chain are essential for NF-κB activation

Mitochondria can produce ROS via autooxidation of molecular O_2 at complex I or complex III of the respiratory chain. We tested the dependence of NF-κB activation on mitochondrial respiratory chain activity. Pretreatment of C2C12 myotubes with rotenone, an inhibitor of complex I of the respiratory chain, inhibited NF-κB activation by TNF-α in a dose-dependent manner (Fig. 1A). This action of rotenone was confirmed in rat primary myotubes (Fig. 1B). Amytal, another complex I inhibitor, had similar inhibitory effects on TNF-α action (Fig. 1C). On the other hand, antimycin A, an inhibitor of complex III, enhanced the activation of NF-κB by TNF-α (Fig. 1A), whereas thenoyltrifluoroacetone (TTFA, 50 μ*M*), an inhibitor of complex II, had no apparent effect (data not shown). These results suggest that ROS generated by complex I of the mitochondrial respiratory chain are essential for TNF-α activation of NF-κB.

PKC is activated by TNF-α

PKC undergoes oxidative activation in neural tissue (Palumbo *et al.*, 1992; Klann *et al.*, 1998) and epidermal cells (Larsson and Cerutti, 1989) and therefore was tested as a possible target for TNF-α/ROS signaling. Under control conditions, basal PKC activity measured in C2C12 myotubes was 14.0 pmol/min per mg protein ± 8.2 SE. This level of activity was relatively low compared to rat hippocampus homogenates that were used as positive controls (20.5 pmol/min per mg protein ± 3.1), and a lower level of PKC was confirmed by Western blot analysis (data not shown). Initial time course studies indicated that basal PKC activity undergoes a transient increase within 15 min of exposing myotubes to 3 ng/ml TNF-α (Fig. 2, left panel), an effect not inhibited by adding 1,000 U/ml superoxide dismutase (SOD), 1,000 U/ml catalase, or SOD + catalase

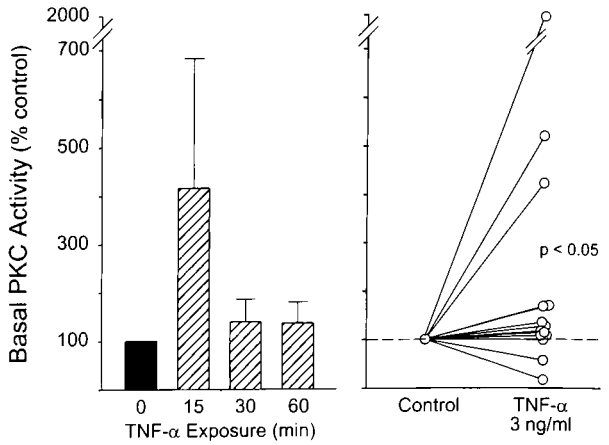

FIG. 2. TNF-α activation of PKC. *Left panel:* Data depict average changes in PKC activity (±SE) of C2C12 myotubes exposed to 3 ng/ml TNF-α for 15–60 min (hatched bars) relative to control myotubes (solid bar); $n = 7$/time point. *Right panel:* Changes in PKC activity of myotubes exposed to 3 ng/ml TNF-α for 15 min; increased PKC activity was observed in 11 of 14 trials (overall significance $p < 0.05$ by Wilcoxon Signed Rank test).

to the culture medium ($p < 0.72$; data not shown). Averaged over all groups, TNF-α was found to increase PKC activity by 196% (±35; $p < 0.05$) relative to control (Fig. 2, right panel). In contrast to basal activity, stimulated PKC activity was 34.6 pmol/min per mg protein (±20.9) under control conditions (37.9 ± 8.3 in hippocampal homogenates) and was unaffected by TNF-α exposure.

Signal transduction mediators that do not mediate TNF-α activation of NF-κB in myotubes

TNF-α can activate both the neutral and acidic sphingomyelinase (SMase) to produce ceramide (for review, Hannun, 1994); in turn, ceramide can mediate activation of NF-κB (Machleidt *et al.*, 1994; Kitajima *et al.*, 1996; Quillet-Mary *et al.*, 1997) by stimulating mitochondrial ROS production through a Ca^{2+}-dependent mechanism (Quillet-Mary *et al.*, 1997). We found that neither synthetic ceramides (C_2- or C_6-ceramide) nor SMase activated NF-κB (Fig. 3, C_6-ceramide data not shown). Similarly, pretreatment of myotubes with the SMase inhibitor oleoylethanolamine (NOE) also failed to block activation of NF-κB by TNF-α (Fig. 3).

Probes that alter the regulation of intracellular Ca^{2+} had no detectable effects in our sys-

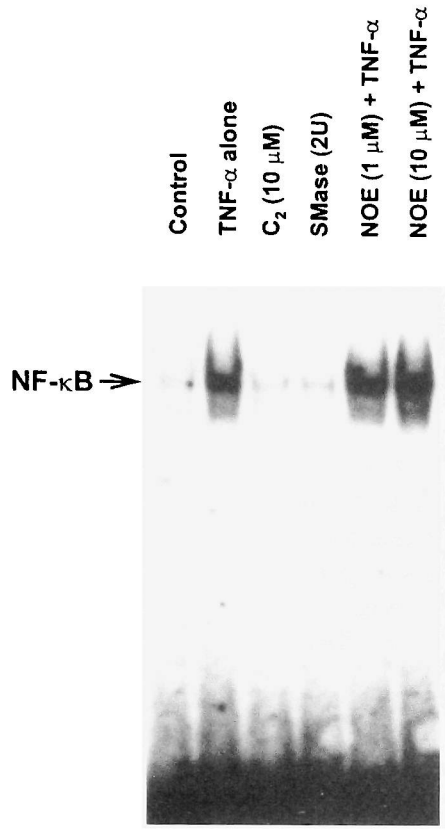

FIG. 3. Effects of synthetic ceramide, SMase, and the SMase inhibitor NOE on NF-κB activation. A representative autoradiograph from three experiments is shown. C2C12 myotubes were treated with 3 ng/ml TNF-α, C_2-ceramide, or SMase for 30 min. NOE pretreatment for 30 min was followed by the addition of 3 ng/ml TNF-α for 30 min.

tem. Ruthenium red inhibits Ca^{2+} release from the sarcoplasmic reticulum (SR) and Ca^{2+} uptake by mitochondria; caffeine stimulates SR Ca^{2+} release; nifedipine inhibits influx of extracellular Ca^{2+} through L-type Ca^{2+} channel (Fleischer and Inui, 1989). As shown in Fig. 4, none of these interventions altered activation of NF-κB by TNF-α.

We also examined the possible involvement of PLA_2 (Liochev and Fridovich, 1997; Thommesen *et al.*, 1998). In our system, NF-κB was not activated by melittin, a stimulator of PLA_2 (Shier, 1979), nor did PLA_2 inhibitors manoalide (Glaser and Jacobs, 1986) or aristolochic acid (Vishwanath *et al.*, 1988) alter TNF-α/NF-κB signaling (Fig. 5).

NO can either facilitate or inhibit NF-κB activation (Lander *et al.*, 1993; Peng *et al.*, 1995; Matthews *et al.*, 1996). Neither response was seen in our myotubes. Pretreatment with the nitric oxide synthase (NOS) inhibitor *N*-nitro-L-arginine did not alter TNF-α activation of NF-κB nor did the NO donor spermine NONOate activate NF-κB (Fig. 6).

DISCUSSION

ROS have been recognized as mediators of intracellular signal transduction. Although TNF-α/NF-κB signaling is known to be ROS sensitive, the importance of ROS in NF-κB activation appears to be cell type-specific. The present study establishes ROS generated by mitochondria as a potent and essential signal for NF-κB activation in skeletal muscle myocytes.

Mitochondria-generated ROS include the superoxide anion radical, which is converted to H_2O_2 by Mn-superoxide dismutase (Mn-SOD),

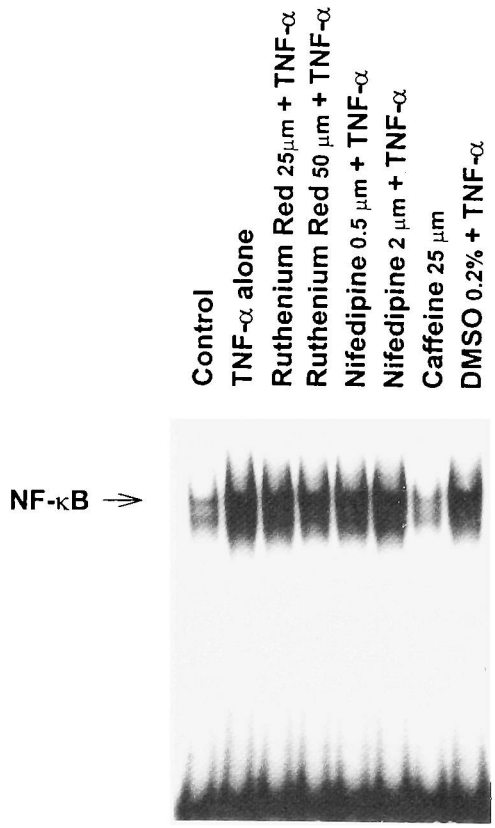

FIG. 4. Effects of Ca^{2+} probes on NF-κB activation. C2C12 cells were pretreated with ruthenium red, nifedipine, or vehicle DMSO for 30 min followed by 3 ng/ml TNF-α treatment for 30 min. Caffeine treatment was carried out for 60 min.

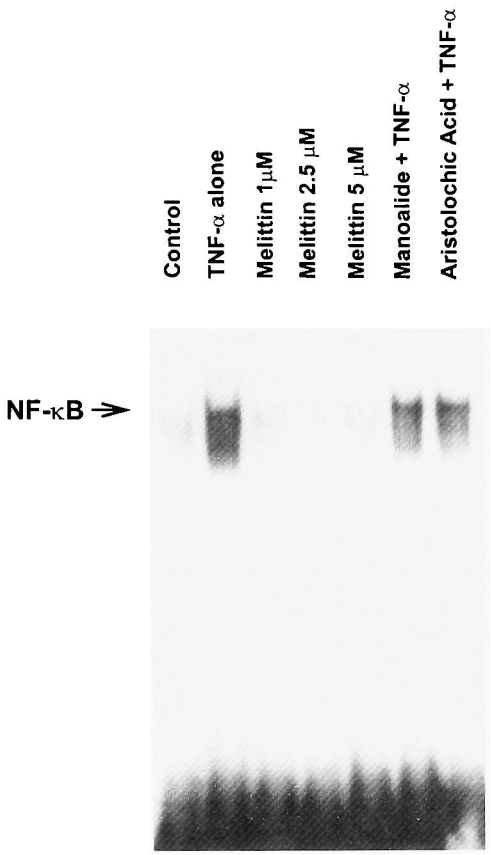

FIG. 5. Effects of PLA_2 activator and inhibitors. C2C12 cells were treated with 3 ng/ml TNF-α or melittin for 30 min. Pretreatment of 2 μM manoalide or 200 μM aristolochic acid for 30 min was followed by 3 ng/ml TNF-α treatment for 30 min.

and hydroxyl radical, which is produced by iron-dependent radical reactions of superoxide anion radical and H_2O_2 (Fridovich, 1978). Our previous data show that exogenous H_2O_2 activates NF-κB in muscle cells but its action is less complete and takes longer to occur than TNF-α (Li *et al.*, 1998). The present study further strengthens the apparent role of ROS in TNF-α/NF-κB signaling.

Our results suggest that increased ROS production may have a profound impact on skeletal muscle biology by activating NF-κB. NF-κB is a transcription factor that regulates a number of genes involved in immune response, cell growth and cell death (Baeuerle and Baltimore, 1996; Karin, 1998). Although the role of NF-κB in muscle biology is not well defined, evidence is beginning to emerge that NF-κB is a key mediator of TNF-α-induced muscle protein degradation (unpublished data). Animal studies have demonstrated that antioxidants can prevent TNF-α-induced muscle loss (Buck and Chojkier, 1996). Our results suggest that the mechanism of antioxidant action involves inhibition of NF-κB activation by TNF-α/ROS signaling.

As in nonmuscle cells (Chen *et al.*, 1995), NF-κB activation in myotubes involves degradation of I-κB, the inhibitory protein of NF-κB, by the ubiquitin–proteasome pathway (Li *et al.*, 1998). Degradation of I-κB requires phosphorylation of the protein by a complex cascade that includes NF-κB-inducing kinase (NIK), I-κB kinase α (IKKα), and I-κB kinase β (IKKβ) (reviewed by May and Ghosh, 1998). A possible mechanism whereby ROS could activate NF-κB is by altering the activity of one or more enzymes within this cascade through thiolation or oxidation of SH groups (Flohe *et al.*, 1997). The present data suggest that PKC could function as the target of ROS action.

TNF-α stimulated the basal activity of PKC but not the Ca^{2+}- and phospholipid-stimulated

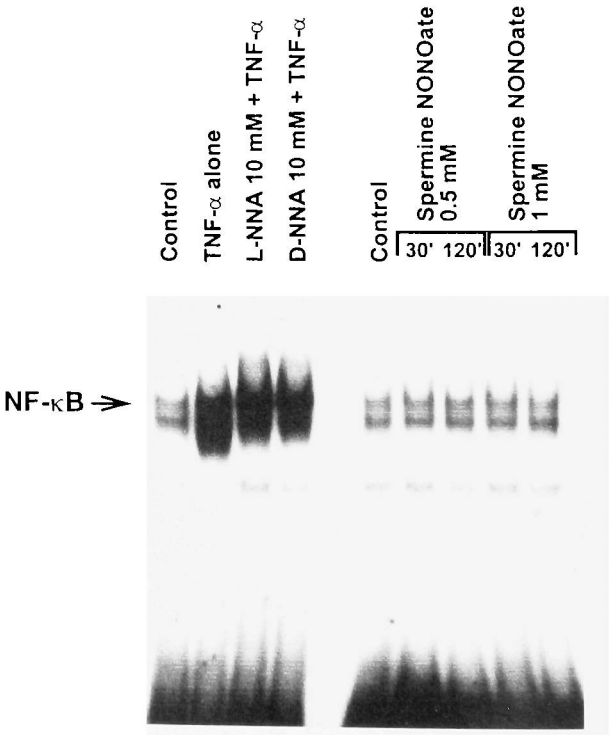

FIG. 6. Effects of a NOS inhibitor and a NO donor. C2C12 myotubes were treated with *N*-nitro-L-arginine (LNN-arginine) or *N*-nitro-D-arginine (DNN-arginine) for 60 min followed by 3 ng/ml TNF-α treatment for 30 min. Spermine NONOate treatment was for 30 or 120 min.

activity. These results are consistent with the observation by Gopalakrishna and Anderson (1989) that oxidative activation of PKC is independent of Ca^{2+} or phospholipid in vitro and may involve selective oxidative modification of the regulatory domain. PKC activation is transient in our system and peaks at an earlier time point than NF-κB activation, which is also transient (Li *et al.*, 1998). The observations are consistent with the assertion that PKC activation is indirectly involved in the phosphorylation of I-κB (Steffan *et al.*, 1995), a step required for the degradation of I-κB and release of NF-κB (Beg *et al.*, 1993). The inability of exogenous SOD and catalase to inhibit PKC activation likely reflects the fact that SOD and catalase are large proteins (131,200 and 240,000 MW, respectively) that are unlikely to cross cellular membranes. Thus, the externally applied antioxidant enzymes could not influence intracellular events directly.

In contrast to mitochondria and PKC, we have no evidence to suggest that ceramide, Ca^{2+}, PLA_2, or NO mediate the TNF-α/NF-κB signaling pathway in differentiated myotubes. These results again demonstrate the individuality of TNF-α signaling in muscle.

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